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Award Number: DAMD17-99-1-9004

TITLE: A Unifying Theory of Prostate Cancer

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REPORT DATE: March 2000

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	March 2000	Annual (15 Feb 99 - 14 Feb 00)

4. TITLE AND SUBTITLE
A Unifying Theory of Prostate Cancer

5. FUNDING NUMBERS
DAMD17-99-1-9004

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*
The purpose of this project is to determine the biological basis of the many unique features of prostate cancer,including high incidence, multifocal origin, zonal specificity,and resistance to chemotherapy. We proposed that these characteristics are the result of dysregulated, albeit wild-type, p53 in prostatic epithelial cells. We determined that p53 is not induced in cells derived from either the central zone (resistant to cancer) or the peripheral zone (high frequency of cancer) in response to ionizing radiation. Since the frequency of premalignant lesions is equivalent in these zones, this finding suggests that genomic instability resulting from dysfunctional p53 predisposses both zones to cancer, but that development of invasive cancer in the central zone is limited by unknown factors. We also determined that p53 is not irreversibly nonfunctional in prostatic cells. Inhibition of RNA transcription or of nuclear export led to increased levels of p53 and increased levels of its transcriptional targets, p21 and mdm2. Therefore, mechanisms responsible for upregulation and activation of p53 protein are intact in prostatic epithelial cells. The deficiency, then, apparently lies in the inability of these cells to respond to signals sent by certain DNA-damaging agents, such as ionizing radiation. We recently found that ultraviolet irradiation, in contrast to ionizing radiation, upregulates and activates p53. This finding demonstrates that the pathway leading to induction of p53 is in fact intact in prostatic epithelial cells, but is not triggered by ionizing radiation. Determining the molecular pathways by which these cells recognize and respond to DNA damage will be extremely relevant to prevention of prostate cancer as well as to treatment.

14. SUBJECT TERMS
Prostate Cancer

15. NUMBER OF PAGES
81

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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Donna M. Peehl 3/9/00

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INTRODUCTION

Prostate cancer is characterized by a number of unique features, including high incidence, multifocal origin, zonal specificity, limited response to androgen ablation, and resistance to chemotherapy (Abbas and Scardino, 1997). Our goal is to determine the biological basis of these properties. Preliminary studies led us to propose that at least some of these characteristics are the result of dysregulated, albeit wild-type, p53 in normal human prostatic epithelial cells and in a majority of prostatic adenocarcinomas. We had noted that p53 was not induced in primary cultures of prostatic epithelial cells in response to DNA-damaging agents such as γ-irradiation (Girinsky et al., 1995). The p53 protein is known to be a key regulator of cell cycle arrest and/or apoptosis (Wiman, 1997). Therefore, in the absence of p53 induction, cell cycle arrest or apoptosis did not occur and DNA damage presumably accumulated. Induction of growth arrest or apoptosis by p53 in response to DNA damage is considered to contribute to the ability of p53 to function as a tumor suppressor gene. Therefore, dysregulated p53 would lead to genetic instability. Our proposed research addresses two questions: what is the basis of dysregulated p53 in human prostatic epithelial cells, and, can p53-mediated cell arrest or apoptosis be restored in these cells?

BODY

Our first designated task was to determine the biological consequences of lack of p53-mediated, G1-cell cycle arrest in prostatic epithelial cells. As part of this task, one of our first projects was to expand our studies to include a series of epithelial cell strains derived from the normal central zone of the adult prostate. Our previous studies had focused primarily on normal epithelial cells derived from the peripheral zone of the prostate. The peripheral zone is the site of origin of the majority of cancers that occur in the prostate, whereas the occurrence of cancers in the central zone is quite rare (McNeal, 1969).

In our prior work, we had reported that none of the cell strains derived the the normal peripheral zones of 7 separate individuals had showed significant induction of p53 in response to DNA-damaging agents (γ-irradiation, hypoxia, or chemicals) (Girinsky et al., 1995). Our initial studies with cell strains derived from the normal central zone were limited, but we had observed that 2 of the 5 cell strains of this type appeared to show some induction of p53 in response to DNA-damaging agents (Girinsky et al., 1995). **We wondered whether central zone cells, unlike peripheral zone cells, retained functional p53; was this the basis for the relative resistance of the central zone to the development of cancer?**

We established 8 additional primary cultures of epithelial cells derived from the central zone and exposed them to 6 Gy of γ-irradiation. Relative levels of p53 protein at times 0, 3, 6 and 24 hours after irradiation were evaluated by immunoblot analysis. None of the cell strains derived from the central zone showed an induction of p53 protein in response to γ-irradiation. These results, then, were similar to those that we had previously found with cells derived from the

peripheral zone. We concluded that p53 was dysfunctional in the central zone as well as in the peripheral zone, and that differences in p53 activity therefore could not explain the differential susceptibilities of the two zones to the development of cancer. However, it is worth noting that prostatic intraepithelial neoplasia (PIN), considered to be the premalignant precursor of invasive cancer in the prostate, occurs at equivalent frequencies in the central and peripheral zones (McNeal and Bostwick, 1986). Lack of p53 activity might therefore relate to the high rate of PIN in both the central and peripheral zones of the prostate, while other unknown factors limit progression of dysplasia to invasive cancer in the central but not the peripheral zone.

Another element of task 1 was to investigate the effects of cellular differentiation on p53 induction. Other investigators have reported that the differentiated status of other cell types, such as keratinocytes, affects levels of p53 and ability to repair DNA damage (Li et al., 1997). There are three recognized subtypes of cells in the prostatic epithelium: basal cells, secretory cells and neuroendocrine cells. Each of these subtypes is characterized by expression of a particular pattern of markers. Examples include expression of keratin 5 in the basal cells, keratin 18 and prostate-specific antigen (PSA) in secretory cells, and chromogranin A in neuroendocrine cells. The exact relationship of these cells is not worked out, but the current popular view is that basal cells include the progenitor (stem?) cells that give rise to either secretory or neuroendocrine cells by alternate pathways of differentiation, presumably triggered by different stimuli (Bonkhoff et al., 1994).

Methods to isolate, promote or maintain these different lineages of prostatic cells in vitro are not well worked out. Specific factors or conditions to maintain basal, secretory or neuroendocrine cell populations are not clearly identified, and markers to distinguish the lineages have not been plentiful. Nevertheless, we have some knowledge of the former from previous studies, and the latter has been improved by recent developments.

From past work, we believe that our primary cultures of normal prostatic cells most closely resemble basal cells when grown in our standard culture conditions. Treatment with retinoic acid promotes a phenotype which more closely resembles secretory epithelial cells (Peehl et al., 1993). High levels of cyclic AMP, on the other hand, promote a phenotype reminiscent of neuroendocrine cells. Previously, our ability to confirm the similarity of cultured cells to basal, secretory or neuroendocrine cells was limited by a relative paucity of markers associated with each lineage. Now, thanks to the efforts of Dr. Alvin Liu at the University of Washington, a panel of antibodies against CD antigens is available to evaluate the basal or secretory nature of cultured prostatic epithelial cells. To establish these panels of antibodies, Dr. Liu localized reactivity of >100 anti-CD antibodies to specific cells in the prostate by immunohistochemistry. He identified a subset of antibodies that reacted specifically with basal cells, another subset that reacted specifically with secretory cells, and another set that reacted with both types of cells (Liu et al., 1997). Other antibodies reacted specifically with cells in the prostatic stroma. In a recent study, Dr. Liu found that stromal cells cultured from prostatic tissues have an interesting phenotype, as revealed by reactivity with anti-CD antibodies. Surprisingly, cultured stromal cells expressed some CD antigens that were present only in the epithelium in tissues, or were

expressed by endothelial or blood cells rather than by prostatic stromal cells in vivo. Additional studies of this nature in the future will undoubtedly afford unique insights into the nature of cultured cells.

Dr. Liu has shared many of his anti-CD antibodies with us, and we have begun to evaluate the expression of these markers in our cultured prostatic epithelial cells. As hinted at previously with our limited array of cell type-specific antibodies, individual cells are neither entirely basal-like or secretory-like. Rather, some markers of each lineage are co-expressed. We don't yet understand the significance of our findings and certainly this is a topic beyond the subject of this grant, but nevertheless, these antibodies have provided us with a new tool that will prove useful in our current studies.

Accordingly, we treated primary cultures of prostatic epithelial cells with factors that we believe may change the differentiated status of the cells. These included the deletion of epidermal growth factor (EGF) from the culture medium, or the addition of retinoic acid or 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$]. Deletion of EGF stops proliferation and cellular migration, and causes the cells to form acinar-like arrangements in two-dimensions. Retinoic acid and $1,25(OH)_2D_3$ also inhibit growth and induce morphological changes (Peehl et al., 1993 and 1994) . Previously, we reported that treatment with retinoic acid increased the expression of keratins 8 and 18 in these cells, giving a hint of the induction of a more secretory-like phenotype. We pretreated cells for three days with each of these conditions, then exposed the cells to 6 Gy of γ-irradiation. Relative levels of p53 protein were measured by immunoblot analysis at times 0, 6 and 24 hours after irradiation. Treatment of cells with retinoic acid or $1,25(OH)_2D_3$ alone, or deletion of EGF, did not induce p53. Irradiation of retinoic acid -treated cells also did not increase levels of p53 protein. However, our preliminary results suggest that treatment with $1,25(OH)_2D_3$ resulted in an upregulation of p53 protein in response to irradiation. This is a potentially valuable finding which we will pursue. If true, it will be important to determine the basis for the ability of vitamin D-treated but not untreated cells to upregulate p53 after irradiation. This ability would be very relevant to strategies to develop more effective radiotherapy protocols to treat prostate cancer.

Other studies that we performed during this period related to the goal of task 1 to determine the biological consequences of lack of p53-mediated, G1-cell cycle checkpoint in prostatic epithelial cells. We suggest that, if prostatic cancer cells are incapable of undergoing p53-mediated cell cycle arrest or apoptosis, then drugs that would be expected to be effective against prostate cancer would be those that induce apoptosis via p53-independent mechanisms. Our studies so far bear this out. We tested a number of agents that have been reported to induce p53-mediated apoptosis in certain other types of cells, and found that none induced p53 or apoptosis (Table I). In contrast, the compound brefeldin A (BFA), reported to induce apoptosis in cells by p53-independent pathways (Shao et al., 1996), was a potent inducer of apoptosis in primary cultures of prostatic cancer cells (Wallen et al., manuscript submitted for publication, see appendix). Development of this or related compounds, or identification of

other agents that induce p53-independent death, might be anticipated to be quite effective for chemotherapy of prostate cancer.

Another question relevant to the goals of task 1 is, if γ-irradiation does not block cell cycle progression or induce apoptosis in prostatic epithelial cells, what phenotype is induced? Certainly we know that increasing doses of γ-irradiation reduce clonogenic growth of prostatic epithelial cells (experiments in progress). What is the mechanism which reduces proliferative capacity? There are many possibilities, but the one that we are currently pursuing is the possibility that radiation induces a senescent phenotype in prostatic cells. We did a preliminary study showing that the marker senescence-associated β-galactosidase does correspond with the development of senescence in serially-passaged cultures of prostatic epithelial cells (Choi et al., in press). Then, in collaboration with the laboratory of Dr. Joyce Slingerland, we examined the molecular pathways involved in the development of the senescent phenotype (Sandhu et al., manuscript submitted for publication, see appendix). As has been found in other types of cells, $p16^{INK4A}$ was increased in conjunction with senescence. Interestingly, neither p53 or p21 increased in senescent prostatic epithelial cells, in contrast to the increase in these proteins seen in senescent fibroblasts. Here, then, is yet another instance of the lack of participation of p53 in a critical growth-regulatory process in prostatic epithelial cells. Using senescence-associated β-galactosidase and increased p16 as markers, we are now ready to determine whether γ-irradiation limits growth of prostatic epithelial cells by inducing senescence by a non-p53-mediated process.

Task 2 was to attempt to restore p53 activity and the G1-checkpoint to prostatic epithelial cells. We have achieved this goal by using three diverse approaches:

Treatment with inhibitors of RNA transcription

The first approach involved treating prostatic epithelial cells with actinomycin D or DRB, two well-known inhibitors of RNA transcription. The rational was to inhibit the transcription of the mdm2 gene, whose product binds the p53 protein and targets p53 for ubiquitin-mediated degradation (Prives, 1998). This process is considered to be the key mechanism causing the rapid turnover of the p53 protein, keeping its levels low in the absence of stress. Whereas p53 levels are mainly regulated by this post-transcriptional mechanism, levels of mdm2 are mainly regulated at the transcriptional level (An et al., 1998).

We found that p53 levels were indeed upregulated by exposure of prostatic epithelial cells to actinomycin D or DRB (Table II). Treatment with 1 μM of actinomycin D or 100 μM of DRB resulted in upregulation of p53 protein by 6 hr. This finding demonstrated for the first time that p53 was capable of being upregulated in prostatic epithelial cells.

However, the nature of this approach did not allow us to determine whether p53 was capable of functioning after upregulation. Because p53 is a transcriptional factor, the presence of

actinomycin D or DRB blocked the transcriptional activation of typical targets of p53 such as p21. Therefore, we sought other approaches that might upregulate p53 and also permit us to investigate its functions and biological effects.

Treatment with leptomycin B

To that end, we tested the effects of leptomycin B (LMB), an anti-fungal agent that has been found to be a unique inhibitor of the cell cycle of mammalian cells. Recently, LMB was found to inhibit specific protein and RNA export from the nucleus to the cytoplasm. LMB achieves this by interacting directly with CRM1, a receptor that mediates the export from the nucleus of viral and cellular proteins containing leucine-rich nuclear export signals. Lain et al. (1999) found that addition of LMB to cultures of normal human fibroblasts led to accumulation of p53 and p53-responsive genes in the nuclei of these cells. A nuclear export signal has been described in the mdm2 gene as well as in the p53 gene, suggesting that export of p53 from the nucleus is mediated by the CRM1 exportin. Presumably, LMB upregulates nuclear p53 protein by preventing mdm2- mediated transport of p53 to the cytoplasm for ubiquitin-mediated degradation.

We treated prostatic epithelial cells with LMB for 4 hours and measured cellular levels of p53 protein by immunoblot analysis (Figure 1, see appendix). In untreated cells or cells exposed to γ-irradiation, p53 protein levels remained low. In contrast, p53 levels were significantly elevated by treatment with LMB at 20 nM, or the proteasome inhibitor MG132 at 25 μM. By immunocytochemistry, we also demonstrated the nuclear accumulation of p53 in the presence of LMB.

In additional studies, we found that short-term exposure (2 to 4 hours) of cells to LMB induced long-term elevation of p53 (Figure 2, see appendix). Other studies were performed to test the biological effects of this long-term elevation of p53. Cell-cycle analysis by flow cytometry demonstrated a G1-arrest after treatment with LMB (Figure 3, see appendix). Results from growth assays further demonstrated that exposure to LMB causes proliferative arrest. We treated prostatic epithelial cells for 4 hours with LMB (at which time p53 was determined to be upregulated), then washed away the LMB and tested the ability of the cells to proliferate in clonal growth assays. Treatment with as little as 0.25 ng/ml of LMB for 4 hours resulted in a 40% decrease in clonal growth ability, and treatment with 0.5 ng/ml of LMB completely abrogated subsequent proliferative potential. Additional experiments indicated that the effects of LMB were irreversible. When cells were treated with 0.25 ng/ml of LMB for 4 hours, then were permitted to recover for 24 hours in LMB-free medium before testing for proliferative potential, clonal growth was reduced even further.

LMB, then, like actinomycin D or DRB, caused an accumulation of cellular p53 in prostatic epithelial cells. Based on the mechanism of action of LMB, we expected that p53 accumulation in response to LMB would occur as a result of an increase in the half-life of the p53 protein. This indeed was the case, as shown in Figure 4 (see appendix). In untreated prostatic epithelial

cells, the half-life of p53 was between 30 and 60 minutes. In LMB-treated cells, the half-life was 60 to 120 minutes.

Use of LMB, rather than the transcriptional inhibitors, gave us the opportunity to investigate the functional effects of p53 upregulation. We examined the effects of upregulation of p53 on the p21 and mdm2 genes, well-known targets of p53's transcriptional activation. Immunoblot analysis (Figure 5, see appendix) revealed that p21 and mdm2 were upregulated in correspondence with an increase in p53 levels. This suggested that upregulation of p53 by LMB resulted in active p53, as indicated by an increase in p53's targets, p21 and mdm2.

In other types of cells, upregulation of p53 and p21 result in cell cycle arrest and/or apoptosis. Our experiments to evaluate these possibilities in prostatic epithelial cells suggest that exposure to LMB and subsequent upregulation of p53 and p21 are not sufficient to induce apoptosis.

Treatment with ultraviolet irradiation

Ultraviolet irradiation induces p53 in certain cells which are defective in γ-irradiation –induction of p53. We exposed normal prostatic epithelial cells to increasing doses of ultraviolet B (UVB)-irradiation and evaluated p53 levels by immunoblot. It was evident that a robust induction of p53 occurred, with an increase seen at 3 hours and peaking at 8 hours. These same cells showed no induction of p53 in response to γ-irradiation. Furthermore, induction of p53 was accompanied by upregulation of p21 and mdm2, two targets of p53 transcriptional activation. This was evidence that activation of p53 accompanied stabilization and upregulation.

Regulation of p53

This result, then, provides us with a valuable key to dissecting out the regulation of p53 in prostatic epithelial cells. Both UVB and γ-irradiation induce DNA damage, yet only UVB provides a signal that stabilizes and activates p53 in prostatic epithelial cells. What is the basis of this differential effect? This will be the subject of our application for the Dual Phase Award Phase II.

KEY RESEARCH ACCOMPLISHMENTS

* determined that p53 protein in normal epithelial cells derived from the central zone of the prostate is not induced in response to DNA damage caused by γ-irradiation

* determined that treatment of prostatic epithelial cells with putative differentiation agents (retinoic acid or vitamin D) did not themselves induce p53 protein.

* Noted in preliminary studies that treatment of prostatic epithelial cells with vitamin D possibly sensitized the cells to induction of p53 by γ-irradiation

* Tested a number of drugs or other compounds and found that only one, brefeldin A, which acts via p53-independent mechanisms, induced apoptosis in prostatic epithelial cells

* Confirmed that senescence-associated β-galactosidase and increased levels of p16, but not p53 or p21, are markers of senescence in prostatic epithelial cells

* showed that p53 protein in prostatic epithelial cells was inducible by treatment with inhibitors of RNA transcription (actinomycin D or DRB)

* demonstrated that treatment of prostatic epithelial cells with leptomycin B, an agent that blocks export of proteins from the nucleus to the cytoplasm, leads to upregulation of p53 protein

* found that p53 protein upregulated by leptomycin B in prostatic epithelial cells was active, as demonstrated by an increase in transactivation targets of p53 (mdm2 and p21)

* observed that induction of active p53 by leptomycin B led to G1-cell cycle arrest and irreversible growth inhibition

* discovered that DNA damage caused by ultraviolet B-irradiation, in contrast to that caused by γ-irradiation, induced p53 in prostatic epithelial cells

REPORTABLE OUTCOMES

Manuscripts

Sandhu, C., Peehl, D.M. and Slingerland, J. $p16^{INK4A}$ mediates Cdk4/6 inhibition in senescent prostatic epithelial cells. Submitted to Cancer Research.

Wallen, E., Sellers, R.G. and Peehl, D.M. Brefeldin A induces p53-independent apoptosis in primary cultures of human prostatic cancer cells. Submitted to Journal of Urology.

CONCLUSIONS

DNA damage induced by γ-irradiation, hypoxia, and certain chemicals does not activate p53 in normal prostatic epithelial cells from the peripheral zone, the main site of origin of prostate cancer. This lack of p53 induction, and therefore lack of cell cycle arrest or apoptosis, may lead to genomic instability and the development of cancer. The central zone of the prostate is relatively immune to cancer. We hypothesized that p53 may be functional in cells from the

central zone, thus protecting these cells from DNA damage and genomic instability. This turned out not to be the case – p53 was not activated in cells from the central zone in response to γ-irradiation, similarly to cells from the peripheral zone. We conclude that maintenance of functional p53 does not occur in central zone cells and therefore cannot be the basis of differential susceptibility to cancer in the central zone versus the peripheral zone. However, since precursor lesions of prostate cancer (PIN) occur at equal frequencies in the central and peripheral zones, we suggest that genomic instability caused by dysfunctional p53 may lead to initiating events in both zones, but cancer progression is limited in the central zone by unknown factors.

One of our goals was to determine whether p53 was irreversibly nonfunctional in prostatic epithelial cells. The experiments that we performed during this period have shown that this is not the case. Treatment of cells with inhibitors of RNA transcription resulted in elevated levels of p53 protein. This was presumably due to the inhibition of transcription of mdm2, the protein which regulates the degradation of p53 protein and whose own levels are regulated by transcription. While these experiments showed that p53 protein could be upregulated in prostatic epithelial cells, the question of whether this elevated p53 protein was functional could not be addressed because inhibitors of transcription block the transactivation properties of p53. Therefore, we tested an alternate means of elevating p53 by treating cells with leptomycin B (LMB), an inhibitor of nuclear transport. Since p53 levels are normally kept low by translocation from the nucleus to the cytoplasm for ubiquitin-mediated degradation, inhibition of nuclear transport should elevate levels of p53 protein. We showed that this indeed occurred in prostatic epithelial cells. Furthermore, this p53 protein was active, as shown by a concomitant increase in p21 and mdm2, two of p53's transcriptional targets. LMB-mediated activation of p53 caused arrest in the G1-phase of the cell cycle, and irreversible loss of proliferative potential.

In conclusion, we now know that mechanisms responsible for the upregulation of p53 protein and activation are intact in prostatic epithelial cells. The deficiency, then, lies in the inability of the cells to respond to signals sent by certain DNA-damaging agents, such as γ-irradiation, to stabilize and upregulate p53 protein. Our most recent result provides a means to identify the factors responsible for this deficiency. We found that ultraviolet irradiation, in contrast to γ-irradiation, upregulates p53. Therefore, this part of the pathway leading to induction of p53 is in fact intact in prostatic epithelial cells, but is not triggered by γ-irradiation. This suggests that the deficiency lies in the ability of prostatic epithelial cells either to recognize or to respond to certain types of DNA damage. Determining the molecular pathways by which prostatic cells recognize and respond to DNA damage will be extremely relevant to prevention of prostate cancer as well as to treatment.

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Table 1: Lack of p53 induction in Prostatic Epithelial Cells

Cell Strain	Factor	p53 Induction
E-PZ-31	Calcium	-
E-CA-16	Calcium	-
E-CA-12	A23187	-*
E-CA-22	A23187	-
E-CA-20	Suramin	-
E-PZ-31	Etoposide	-*
E-CA-22	Etoposide	-
E-CA-16	Methotrexate	-
E-CA-22	Methotrexate	-
E-CA-12	Mitomycin C	-
E-CA-12	Retinoic acid	-
E-CA-16	Vitamin D	-
E-CA-20	TPA	-
E-CA-12	TGF-beta	-
E-CA-12	TNF-alpha	-
E-CA-16	IFN-gamma	-

* Late (24hr) and no p21 induced

Table II: Induction of p53 by inhibitors of RNA transcription

Cell Strain	Factor	p53 Induction
E-CA-20	Act D	+
E-CA-22	Act D	+
E-PZ-26	Act D	+
E-PZ-26	DRB	+
E-CA-20	DRB	+

Figure 1

Induction of p53 in FWpz cells after 4 hour treatment with Leptomycin B and MG132, a proteasome inhibitor, but not by Ionizing Radiation

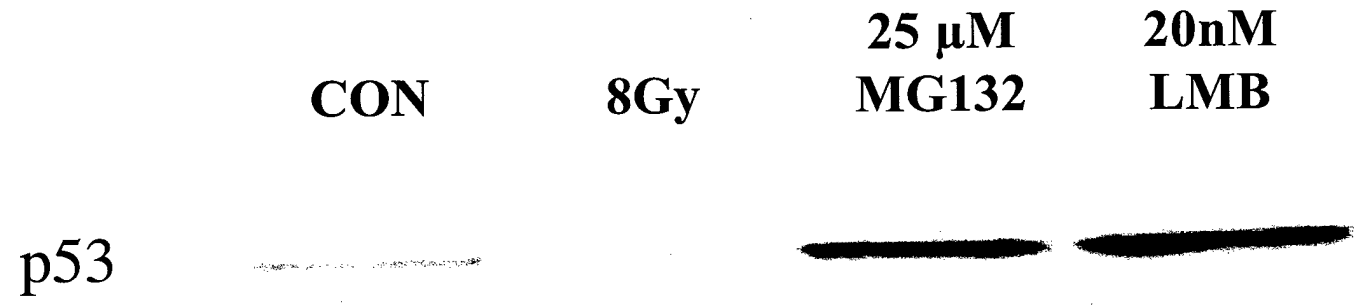
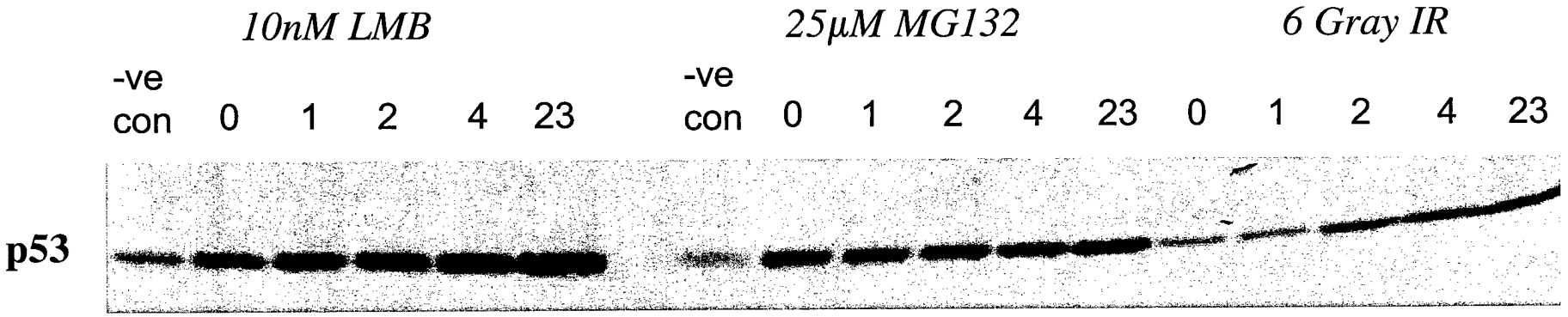


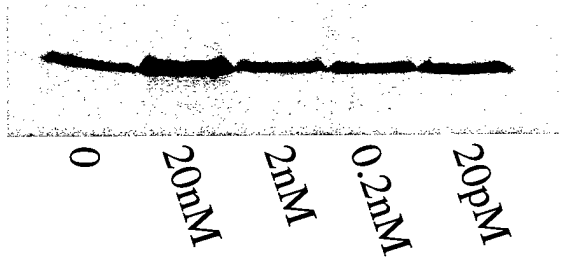
Figure 2

Short term exposure of normal prostate cell strains to Leptomycin B induces long-term elevation of p53 protein

FWpz (4 hour exposure)



EYWpz



2 hour exposure to various concentrations of LMB, harvested 24 hr later

Figure 3

Leptomycin B induces normal Prostate epithelial cells to arrest in the G1 phase of the cell cycle

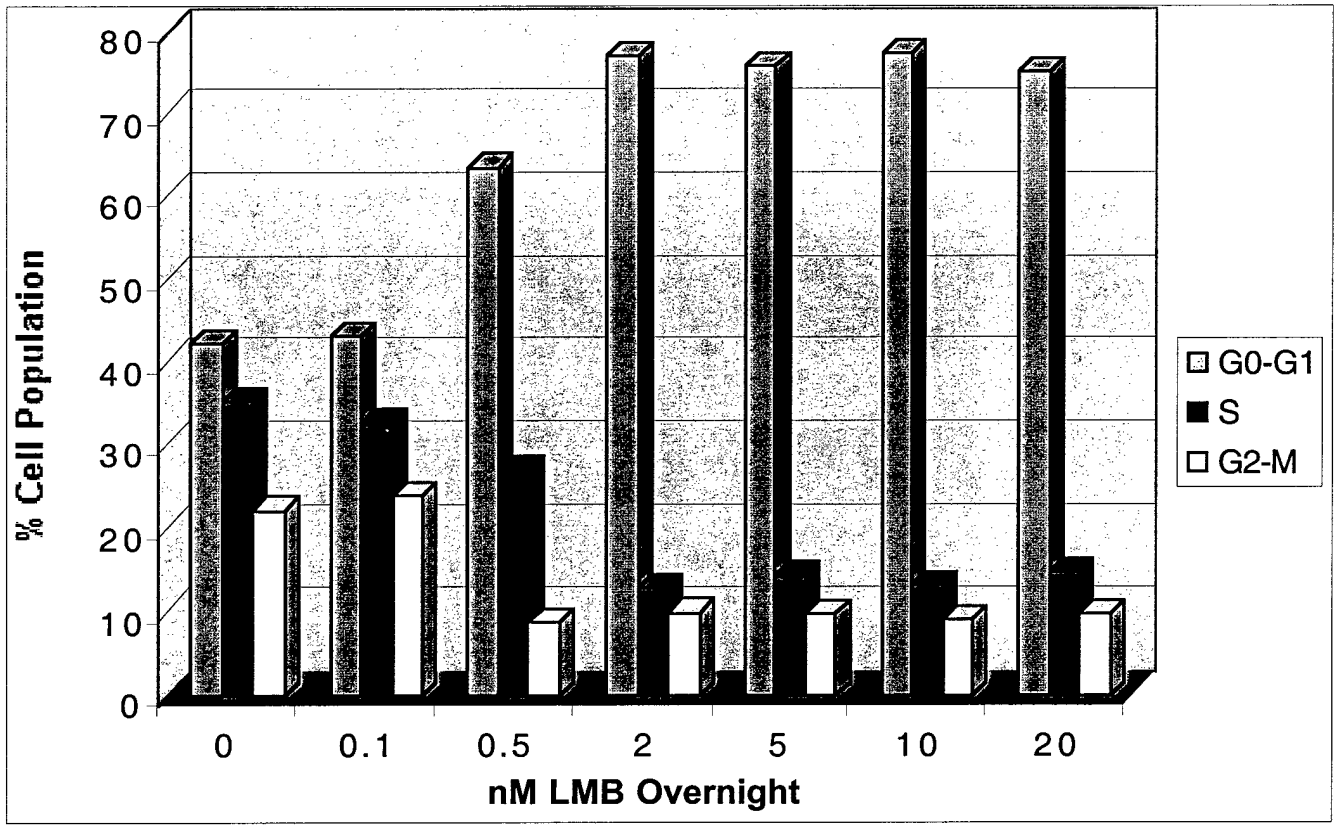
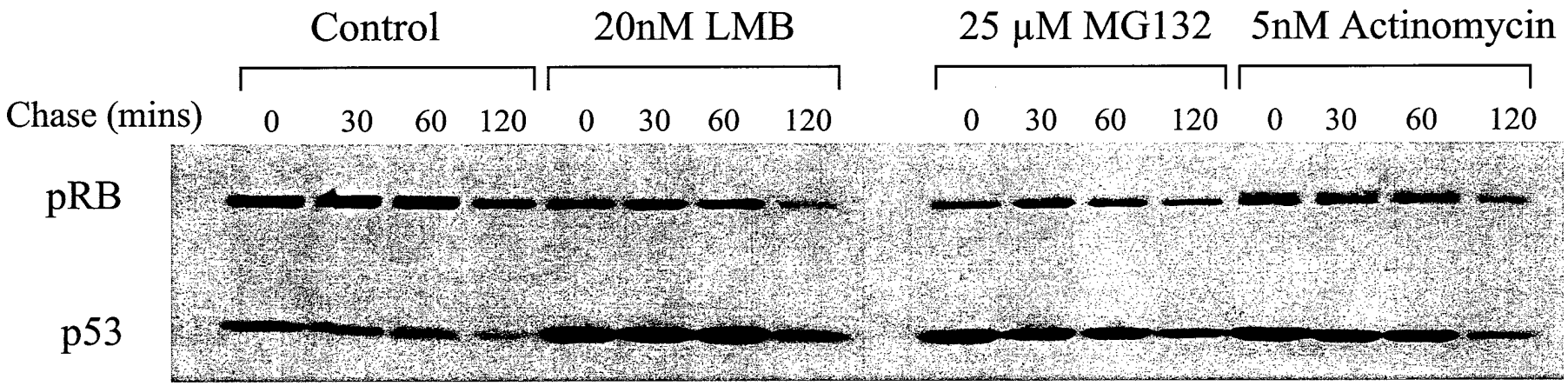


Figure 4

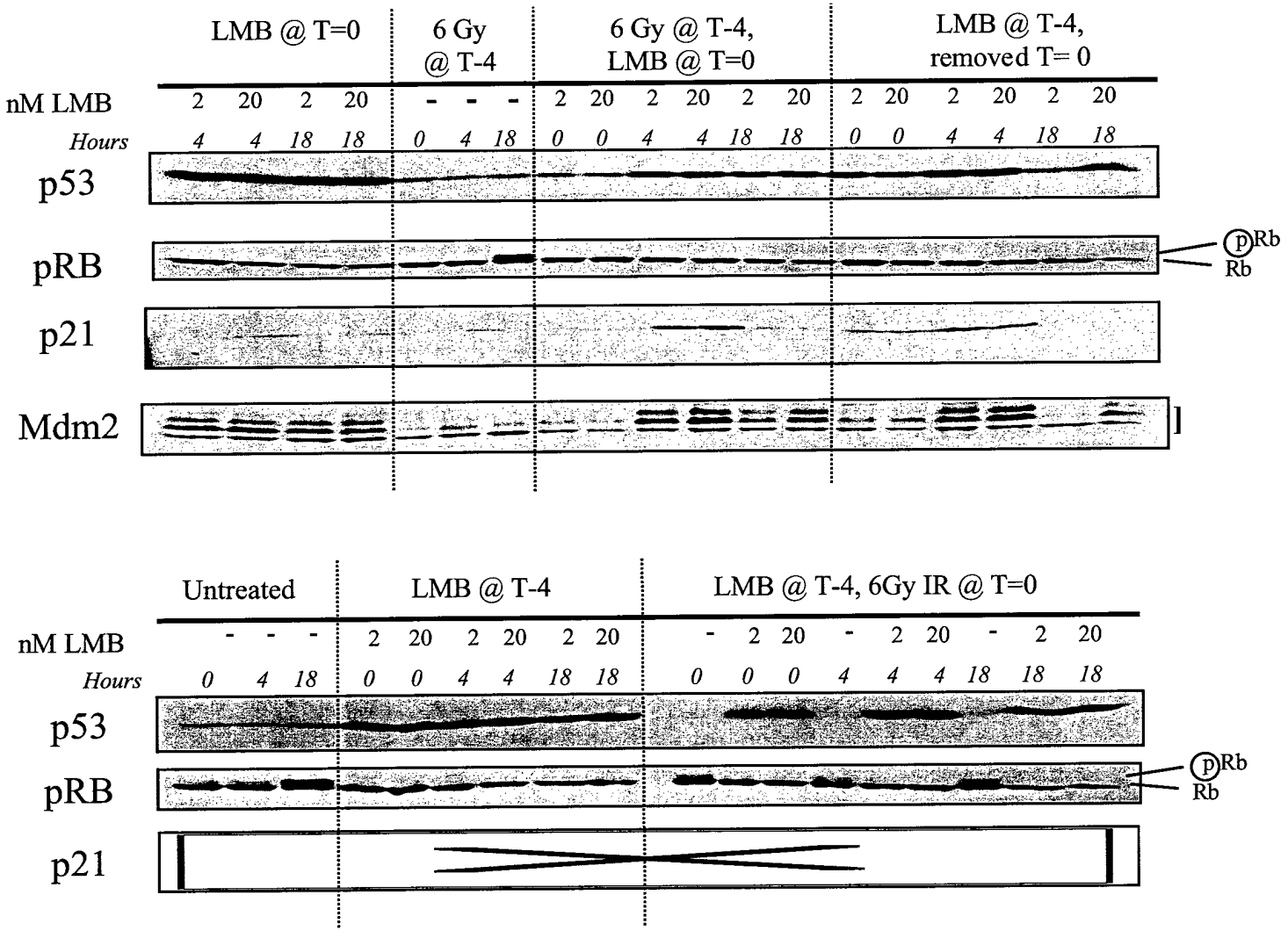
Leptomycin B extends the Half-Life of p53 in FWpz cells



Cells were treated for 4 Hours with Leptomycin B, MG132 or Actinomycin D after which the cells were washed and 40 μg/ml cycloheximide added.

Figure 5

p53 induced by LMB is active transcriptionally



BREFELDIN A INDUCES p53 -INDEPENDENT APOPTOSIS IN PRIMARY CULTURES OF HUMAN PROSTATIC CANCER CELLS

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KEY WORDS: prostate cancer, p53, apoptosis, chemotherapy, brefeldin A

ABSTRACT

Purpose: The objective of this study was to investigate growth-inhibitory and apoptotic activity of the experimental antitumor drug, brefeldin A (BFA), on primary cultures of human epithelial cells derived from prostatic adenocarcinomas.

Materials and Methods: Clonal assays were performed to evaluate the effects of BFA on growth of prostatic cancer cell strains. Loss of cell viability in response to BFA was assessed by trypan blue exclusion. Induction of apoptosis by BFA was evaluated by morphologic criteria, electrophoretic assay of DNA fragmentation, and a cell death ELISA. Immunoblots were used to monitor p53 and pRB expression in response to BFA.

Results: BFA was growth-inhibitory at a half-maximal concentration of 5 ng/ml (18 nM). Morphological manifestations of apoptosis were evident by 24 hours of treatment. Cell viability declined and the cell death ELISA indicated an 18-fold increase in apoptosis in BFA-treated versus untreated cells at 48 hours. DNA fragmentation was also seen at 48 hours. Levels of p53 were not altered by BFA, but pRB was maintained in a hypophosphorylated state by BFA treatment.

Conclusions: BFA is a potent inducer of apoptosis in prostatic cancer cells via a p53-independent mechanism. Cells derived from low- as well as high-grade cancers responded similarly to BFA. Since p53-mediated pathways of apoptosis may frequently be abrogated in prostatic cancer cells, agents such as BFA that induce p53-independent cell death may be promising candidates for chemotherapeutic agents.

In 1999, it was estimated that 179,300 men would be diagnosed with cancer of the prostate, and that 37,000 men would die from the disease.[1] For cancers not entirely confined to the prostate, there are few options for long-term control of the disease. Despite investigations of the roles of known oncogenes and tumor suppressor genes, no unifying explanation of the molecular events involved in prostate cancer initiation and progression has been found, and therefore a molecular target for therapy remains elusive.

Recent advances in the understanding of apoptotic (programmed cell death) pathways have led to investigations of molecular promoters and inhibitors of apoptosis in the prostate. Cell growth and death are likely mediated by the balance of promoters of apoptosis (such as fas, bax, bad, bcl-xs and others) and inhibitors of the process (bcl-2, bcl-xl, jun, abl, mdm-2).[2] These factors exert their cumulative effects in part by modulating the activity of regulators of the cell cycle, such as p53 and pRB.

The p53 tumor suppressor gene product possesses the ability to stop a cell from proceeding through the cell cycle to mitosis in response to DNA damage, until the damage is repaired.[3] If repair is ineffective, p53 may direct the cell to undergo apoptosis. We have found that these functions of p53 appear to be attenuated in normal prostatic epithelial cells or in cells derived from prostatic adenocarcinomas, despite the presence of the wild-type p53 gene.[4] In response to numerous DNA-damaging agents or events (chemicals, irradiation, or hypoxia), primary cultures of prostatic epithelial cells do not induce p53 or undergo cell-cycle arrest in G_1 or undergo apoptosis. Many standard chemotherapeutic drugs exert their effects by activating a p53-dependent pathway of apoptosis.[5, 6] The apparent absence of this pathway in prostatic

epithelial cells, even in those with wild-type p53, may explain why these therapies are ineffective for the treatment of prostate cancer. Prostate cancer cells with mutant p53, which occurs fairly frequently in advanced disease,[7] would also be unresponsive to agents that require p53 for activity.

For this reason, it would be desirable to identify agents which exploit p53-independent pathways of apoptosis in prostate cancer cells. A large in vitro drug screen conducted under the auspices of the National Cancer Institute (NCI) identified brefeldin A (BFA) as an agent which markedly inhibited growth of primary cultures of epithelial cells grown from prostatic adenocarcinomas.[8] BFA showed some specificity for prostate cancer cells, with the half-maximal inhibitory dose for prostate cells about 10- to 100-fold lower than for a panel of cell lines derived from eight other types of malignancies.

BFA, a fungal macrolytic lactone, acts to inhibit intracellular transport by disrupting the vesicular coating process, thereby preventing transport of proteins from the endoplasmic reticulum to the Golgi and causing disintegration of the Golgi complex.[9, 10] Because of this property, BFA has been extensively used as a tool to study mechanisms of protein secretion. Effects of BFA, though, are seemingly not limited to those on the Golgi apparatus. BFA has been shown to cause apoptosis in diverse human cancer cell lines, including those derived from leukemia, colon and prostate cancer.[11-15] The mechanism by which BFA induces apoptosis has not yet been elucidated, but the process appears to be p53-independent.

The purpose of our study was to establish and elucidate possible growth-inhibitory mechanisms, including apoptosis, of BFA on primary cultures of prostatic cell strains derived

from adenocarcinomas of the prostate. Our results show that BFA induced apoptosis by a p53-independent pathway and caused a shift in the phosphorylation state of pRB. Our results support the potential of BFA as a chemotherapeutic agent against prostate cancer.

MATERIALS AND METHODS

Cell culture. Tissue samples were dissected from radical prostatectomy specimens. None of the patients had received prior chemical, hormonal or radiation therapy. Histological assessment was performed by Dr. John McNeal as previously described.[16] Epithelial cells were cultured and characterized as described previously.[17] Four cell strains used in this study were derived from tumors of Gleason grade 3+4 (E-CA-2), 4+3 (E-CA-1, E-CA-4), and 5+5 (E-CA-3). An additional cell strain (E-PZ-5) was derived from histologically normal tissue with no evidence of cancer. Both p53 and pRB genes in these cell strains were wild-type.

Clonal growth assays. Secondary passaged cells were grown to about 50% confluency, then were harvested by trypsinization. Clonal growth assays were initiated by inoculating 200 or 500 cells into each 60-mm, collagen-coated dish[17] containing 5 ml of medium. Growth medium was MCDB 105 (Sigma, St. Louis, MO) supplemented with 10 ng/ml of cholera toxin, 10 ng/ml of epidermal growth factor, 10 μg/ml of bovine pituitary extract, 4 μg/ml of insulin, 1 μg/ml of hydrocortisone, 0.1 mM phosphoethanolamine, 30 nM selenium, 0.03 nM all-trans retinoic acid, 2.3 μM α-tocopherol and 100 μg/ml gentamicin. The sources and preparation of these supplements were previously described.[17]

A stock solution of 1 mg/ml (3.6 mM) of BFA (Sigma) was prepared in 100% ethanol and stored at -20° C. Dilutions were made in media and the ethanol concentration was kept constant at 0.01% in control and experimental media. After incubation in a humidified atmosphere of 5% CO_2/95% air at 37°C for 10 days without feeding, the cells were fixed in 10% formalin and

stained with crystal violet.[17] An Artek image analyzer (Dynatech, Chantilly, VA) was used to measure the total area of each dish covered by cells, which is directly proportional to cell number.[18] Triplicate dishes were tested for each control and experimental variable, and each experiment was performed twice. The Student's t-test was used to evaluate significance.

Cell viability. Loss of cell viability was assessed by the trypan blue exclusion method. Cells treated with or without BFA were harvested by trypsinization. After incubation in 0.04% trypan blue (Sigma) for 4 minutes, cells were counted under a hemocytometer. The number of cells which retained the dye (nonviable) and the total cell number were noted.

Detection of apoptosis. Induction of apoptosis by BFA was evaluated by two methods. In the first assay, DNA laddering was monitored. Cells were treated with or without BFA and DNA was extracted at 0, 8, 24 and 48 hours.[19] Briefly, 10^6 cells were collected at each time point and pelleted in an Eppendorf tube. The cell pellet was suspended in 1 ml of 0.02% EDTA in buffered saline, then the cells were pelleted again. TE lysis buffer (0.25% NP-40 in TE buffer, pH 8.0) (35 µl) and Rnase A (10 µl of a 10 mg/ml stock) were added to each tube and the cells were suspended by gentle vortexing. After incubation at 37° C for 20 minutes, 5 µl of proteinase K (from a 20 mg/ml stock solution) were added to each tube. Following incubation for 20 minutes at 37° C, aliquots of 25 µl each were mixed with loading buffer and analyzed by electrophoresis on a 1.8% agarose gel run at 40 V for 4 hours. DNA was visualized by staining with ethidium bromide and photographed under UV light.

Apoptosis was also evaluated with a cell death ELISA kit (Boehringer Mannheim, Indianapolis, IN) which utilizes a monoclonal antibody against histone to detect DNA fragments in the

cytosolic fraction of lysed cells. Cells treated with or without BFA were harvested and lysed according to the manufacturer's instructions. The samples were transferred into 96-well dishes coated with a mouse monoclonal antibody against histone. After incubation and washing, anti-DNA-peroxidase was added to the wells. The reaction was developed with substrate supplied by the manufacturer and the absorbance of the wells was read at 410 nm. The ratio of the absorbance of the treated cells to the untreated cells was calculated as an enrichment factor, which provides a qualitative assessment of apoptosis.

Immunoblot analysis. Cells were grown to approximately 50% confluency. At time 0, cells were fed media containing 0 or 25 ng/ml of BFA. At times 0, 3, 6, 24, and 48 hours after treatment, cell lysates were prepared by collecting trypsinized cells and solubilizing in lysis buffer (0.1 M Tris-HCL, pH 6.8, 1% SDS, 5% glycerol, 0.005% bromophenol blue, 0.005% pyronine Y and 1% β-mercaptoethanol). Aliquots of 20 µl containing lysate derived from 50,000 cells were loaded into each lane of a polyacrylamide-sodium dodecyl sulfate (PAGE-SDS) gel with a 4.6% stacking gel and a 10% running gel.

After separation of proteins by electrophoresis, the samples were transferred out of the gel onto nitrocellulose membrane, using a Transblot apparatus run at 1.5 mA for 90 minutes. Membranes were blocked overnight with 10% horse serum, then incubated with primary antibodies against p53 (Pharmingen, San Diego, CA; clone DO-1, used at 1:100) or pRB (Pharmingen, clone G3-245, used at 1:1000). Bound antibodies were detected using biotinylated secondary antibody (Vector Laboratories, Burlingame, CA, at 1:4000) and the ABC reagent (Vector Laboratories). TMB membrane reagent (Amresco, Solon, OH) was used to develop the color reaction.

RESULTS

Effect of BFA on cell growth. Clonal assays were used to evaluate the effect of BFA on growth of prostatic cancer cells. BFA was first tested at concentrations ranging from 0.1 to 100 ng/ml and total growth after 10 days was compared to growth in the absence of BFA. Figure 1 shows the marked decrease in growth which occurred at concentrations of BFA between 1 and 10 ng/ml. At 1 ng/ml, growth was almost 100% of control, whereas with 10 ng/ml, growth dramatically declined to 20% of control. This pattern of inhibition was seen with all four cancer cell strains that were tested, as well as with one cell strain derived from normal tissue.

In order to more precisely determine the concentration of BFA which half-maximally inhibited clonal growth, the cancer cell strain E-CA-4 was tested in a clonal assay with a narrow range of BFA concentrations. In this assay, half-maximal growth inhibition was observed at 5 ng/ml (18 nM) of BFA (Figure 2).

Effect of BFA on cell viability. Cells treated with BFA were incubated with trypan blue in order to evaluate the proportion of nonviable cells in treated versus untreated populations. Viable cells quickly exclude trypan blue, whereas nonviable cells retain trypan blue in their cytoplasm. After 48 hours of treatment with 25 ng/ml of BFA, cells were trypsinized and incubated with trypan blue. The fraction of cells which retained trypan blue was evaluated microscopically with a hemocytometer. Two cell strains were evaluated. Untreated E-CA-1 cells had an average of 11 +/- 3% nonviable cells at 48 hours, while BFA-treated cells averaged 27

+/- 0% nonviable cells. Similarly, nonviable cells averaged 41 +/- 6% in BFA-treated E-CA-4 populations, versus 11+/- 1% in untreated cells.

Morphological appearance of cells after treatment with BFA. The morphological appearance of cells during treatment with BFA was observed microscopically. Morphological changes consistent with apoptosis were seen in treated cells as early as 24 hours after the initiation of treatment, and increased by 48 hours. These characteristic changes included cell shrinkage, nuclear condensation, membrane blebbing, and detachment from the substrate (Figure 3).

DNA laddering in response to BFA. Semi-confluent cultures of E-CA-4 cells were treated with or without 25 ng/ml of BFA. DNA was extracted at times 0, 8, 24 and 48 hours of treatment. Gel electrophoresis demonstrated a DNA laddering pattern of 180 bp subunits, characteristic of apoptosis, at 48 hours after treatment with BFA (Figure 4).

Measurement of relative levels of apoptosis. A cell death ELISA was used to measure relative apoptosis in untreated versus treated cultures. E-CA-4 cells were treated with or without 25 ng/ml of BFA for 48 hours, then were harvested for the cell death ELISA, which detects DNA fragments in the cytosol with an antibody against histone. By this assay, apoptosis was 18-fold higher in BFA-treated cultures compared to untreated cultures.

Induction of p53 by BFA. Immunoblot analysis was used to examine levels of p53 protein during BFA treatment. Cells were treated with or without 10 ng/ml of BFA. At times 0, 3, 6, 24, and 48 hours of treatment, cell lysates were prepared. Proteins were separated by PAGE-SDS and transferred to filters. Monoclonal antibody specific for p53 was used to detect p53 protein expression. Protein levels of p53 in E-CA-2 cells remained low throughout the course of the experiment regardless of the presence or absence of BFA (Figure 5). As a control for the

induction of p53 in these cells, we treated with an inhibitor of RNA transcription (DRB) for 24 hours. As expected, p53 protein levels increased, presumably due to inhibition of transcription of mdm-2, which targets p53 for degradation. This lack of induction of p53 protein by BFA was also seen in E-CA-1 and E-CA-3 cells.

Phosphorylation of pRB. Prostate cancer cells were grown to semi-confluency and fed 3 days before the start of the experiment. At time 0, cells were fed fresh media with or without 25 ng/ml of BFA. Cell lysates were prepared at times 0, 3, 6, 24 and 48 hours. Immunoblot analysis was performed to evaluate expression and phosphorylation of pRB (Figure 6). At times 0, 3 and 6 hours, untreated and treated cells had no immunoreactive pRB band of the size associated with the hyperphosphorylated state. However, at times 24 and 48 hours, untreated cells had bands typical of both hyperphosphorylated and hypophosphorylated pRB. This would be expected of cells entering the proliferative phase in response to feeding fresh medium at time 0. In contrast, lysates from BFA-treated cells exhibited only hypophosphorylated pRB, indicating blockage of cell progression into the proliferative cycle.

DISCUSSION

Substantial evidence for unique growth-inhibitory and apoptotic activity of BFA is accumulating. Although first tested for anti-tumor activity more than 30 years ago, BFA did not demonstrate activity in murine models in use at the time and interest in BFA declined.[8] It is now known that murine cells are relatively resistant to BFA,[20] and promising activity of BFA in in vitro and in vivo models of human cancer has rekindled interest in this compound.[8]

Our interest in BFA was first aroused when we tested this compound in a drug screen for the NCI. In this screen, cells from four prostate cancer cell strains derived from tumors of Gleason grades 3, 4 or 5 were inoculated into 96-well microtiter dishes and exposed to experimental compounds for six days.[21] At the end of this assay, growth was evaluated by the sulforhodamine B assay, which measures total protein.[22] Using this assay, we discovered that BFA was a potent inhibitor of prostate cancer cell growth, with half-maximal growth inhibition at approximately 20 nM.[8] This value is almost exactly the concentration that we found for half-maximal inhibition of clonal growth in the current study.

Furthermore, when compared to a panel of cell lines from other types of human tumors that were evaluated at the NCI, prostate cancer cells were the most sensitive to growth inhibition by BFA. Melanoma cells, the most sensitive of the other types of cancers in the screen performed at the NCI, demonstrated half-maximal growth inhibition with approximately 29 nM of BFA.[8] The potent and differential activities of BFA on prostate cancer cell strains suggest that BFA

might be a particularly effective chemotherapeutic agent for prostate cancer with minimal toxicity to other organs.

Analysis of patterns of growth inhibition in the NCI in vitro cancer agent screen suggests that agents with similar mechanisms of antiproliferative action yield similar patterns of growth inhibition.[23, 24] Unique patterns of activity may therefore indicate potentially novel or unique mechanisms of action. It is intriguing that when this analysis was applied to BFA, a unique pattern of susceptibility that didn't resemble the pattern generated by known cytotoxic agents was noted. Therefore, identifying the mechanism of action of BFA on prostate cells becomes particularly relevant to further development of this or related agents as novel chemotherapeutic agents.

The NCI drug screen was not designed to investigate mechanisms of action of experimental compounds. Therefore, in our current studies, we developed additional assays to further define BFA's activity on prostate cancer cells.

One striking observation regarding BFA is the very steep concentration - effect relationship that has been found in diverse studies. In our assays, we found that 1 ng/ml of BFA produced almost no effect on cell growth, whereas 10 ng/ml of BFA completely inhibited growth. By testing a narrow range of concentrations, we demonstrated that half-maximal growth inhibition occured at 5 ng/ml (18 nM) of BFA. Similarly, inhibitory concentrations of BFA for the prostate cancer cell lines PC-3 and LNCaP were between 10 and 100 nM in the NCI screen [8] and between 10 and 30 ng/ml (36 and 108 nM) in experiments performed with PC-3 and another cancer cell line, DU 145, by various other investigators.[14, 15] This narrow range of effective concentrations

of BFA is not peculiar to prostate cells but was also noted in studies with leukemia and colon carcinoma cell lines.[11]

During the course of the growth assays, it became apparent that BFA induced dramatic morphologic changes in the treated prostate cancer cell strains. These changes were reminiscent of those occurring during the process of apoptosis and included membrane blebbing, shrinkage of cytoplasm, and detachment from the substratum. To further assess the induction of apoptosis by BFA, we measured viability by trypan blue exclusion and found that the percentage of nonviable cells in the population increased significantly by 48 hours after exposure to BFA. The results of a cell death ELISA further indicated that nonviability was due to apoptosis, and that the number of apoptotic cells was enhanced 18-fold after 48 hours of BFA-treatment. Finally, the presence of DNA laddering , a classic manifestation of apoptosis, was demonstrated in treated cells.

The time course of induction of apoptosis in prostate cancer cell strains is reminiscent of that reported for the K562 human leukemia cell line and the HT-29 colon carcinoma cell line, but is much slower than that found for HL60 cells, in which DNA laddering was visible after only 15 hours of BFA-treatment.[11] Interestingly, although BFA induced apoptosis in DU 145 cells,[15] it did not do so in PC-3 cells.[14] BFA-treatment caused growth inhibition and detachment of a majority of PC-3 cells after 72 hours of treatment, but >85% of those cells were still viable and the effects were reversible upon removal of BFA. PC-3 cells were found to be blocked in the G_1 - phase of the cell cycle by BFA; in preliminary studies, we did not find this to be the case for prostatic cancer cell strains (data not shown).

Effective chemotherapy for cancer of the prostate is lacking. We hypothesize that the chemoresistance of prostate cancer may be due in part to an attenuated response of p53 in prostatic epithelial cells. While the exact role of p53 in regulating chemosensitivity is still under investigation[25], in at least some types of cells, p53 enhances chemosensitivity by promoting apoptosis. Mutation of p53 in prostate cancer is seemingly less frequent than in some other cancers and is generally a late event,[26] so loss of p53 activity would not be predicted to play a large role in chemoresistance of prostate cancer. However, we observed that prostate cancer cells with wild-type p53 did not induce p53 or undergo cell arrest or apoptosis in response to DNA-damaging drugs or radiation.[4] This lack of activity of p53, despite the widespead maintainance of wild-type p53 in prostate cancer, may therefore explain lack of activity of chemotherapeutic agents, many of which induce apoptosis through p53-mediated pathways. These observations suggest that effective new therapies for prostate cancer will be based on agents that induce p53-independent pathways of cell arrest or apoptosis.

BFA appears to be one such agent that is capable of inducing p53-independent cell arrest or apoptosis. Treatment of several human leukemia or colon carcinoma cell lines with BFA induced apoptosis in a p53-independent manner.[11] Other investigators have shown that PC-3 cells, which have mutated p53, are growth-inhibited by BFA. We found that apoptosis of prostate cancer cell strains induced by BFA was also p53-independent. After 3 to 48 hours of exposure to BFA, levels of p53 protein remained low in treated and untreated cells.

On the other hand, Mordente et al. showed that BFA-treatment of PC-3 cells modulated phosphorylation of pRB.[14] In their experiments, substantial levels of hyperphosphorylated and

hypophosphorylated pRB were present at 24 hours in treated and untreated cells. Declining levels of hyperphosphorylated pRB were seen after 48 hours of BFA-treatment, and by 72 hours, hypophosphorylated pRB was the main form present in the treated cells. Our results were similar. At 48 hours, both hyperphosphorylated and hypophosphorylated pRB were present in untreated prostate cancer cell strains, whereas only hypophosphorylated pRB was present in BFA-treated cells. Although these results suggest that modulation of pRB is part of the mechanism of action of BFA on prostate cancer cells, DU 145 cells with defective pRB nevertheless underwent apoptosis in response to BFA with no change in the phosphorylation status of pRB.[14]

The potent growth-inhibitory and apoptosis-inducing properties of BFA against prostate cancer cells make this compound an interesting candidate for chemotherapeutic application against prostate cancer. Although activity of BFA in human xenograft models of prostate cancer has not yet been reported, in vivo antitumor activity of BFA has been shown against human melanoma xenografts.[8] Certainly, however, BFA has properties that diminish its appeal as a therapeutic drug. For one, it is poorly soluble in aqueous medium, making clinical application problematic. In addition, the very narrow range of toxic concentrations may make dosing difficult. The slow nature of its action and potential reversibility also are problematic.

Nevertheless, these problems may be overcome or modified as we learn more about the mechanism of action of BFA. In their investigations of the effects of BFA on leukemic and colon cancer cell lines, Shao et al found that BFA potentiated induction of apoptosis by protein kinase C inhibitors.[11] BFA was less enhancing with inhibitors of topoisomerase inhibitors, suggesting

that BFA may act more selectively through the apoptosis pathway mediated by protein kinase C inhibitors than the pathway induced by DNA damage. Particularly exciting is the recent discovery that BFA specifically inhibits a Golgi - associated guanine nucleotide exchange activity for the small GTP-binding protein ADP-ribosylation factor 1 (ARF1).[27] It has been suggested that inhibition by BFA of ARF1 activation by trapping the exchange reaction in a dead-end complex may have profound implications on the development of drugs targeting other exchange factors for small G proteins.[28] The fact that different ARF exchange factors have different sensitivities to BFA may be the basis for the differential sensitivity of cells derived from different organs to BFA. Further studies such as these will identify the molecular mechanisms of BFA activity and contribute to the development of novel therapeutic agents.

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FIGURE LEGENDS

Figure 1. Inhibition of clonal growth by BFA. On day 0, four cell strains derived from cancers (E-CA-1, E-CA-2, E-CA-3 or E-CA-4) and one cell strain from normal tissue (E-PZ-5) were inoculated at 200 or 500 cells per dish into growth medium with the indicated concentrations of BFA. After 10 days of incubation, cells were fixed and stained and total growth was quantitated. For each cell strain, growth in the absence of BFA was set at 100%. Each point represents the average of duplicate experiments, with three dishes per point in each experiment, +/- SEM.

Figure 2. Determination of half-maximal growth-inhibitory dose of BFA. On day 0, E-CA-4 cells were inoculated at 200 cells per dish into growth medium with the indicated concentrations of BFA. After 10 days of incubation, cells were fixed and stained and total growth was quantitated. Growth in the absence of BFA was set as 100%. Each point represents the average of duplicate experiments, with three dishes per point in each experiment, +/- SEM.

Figure 3. Morphological characteristics of apoptosis. Semi-confluent populations of E-CA-4 cells were grown with or without 25 ng/ml of BFA and photographed at 72 hours. Top panel: cells grown without BFA (x200); middle panel: cells grown with BFA (x200); bottom panel: cells grown with BFA (x800).

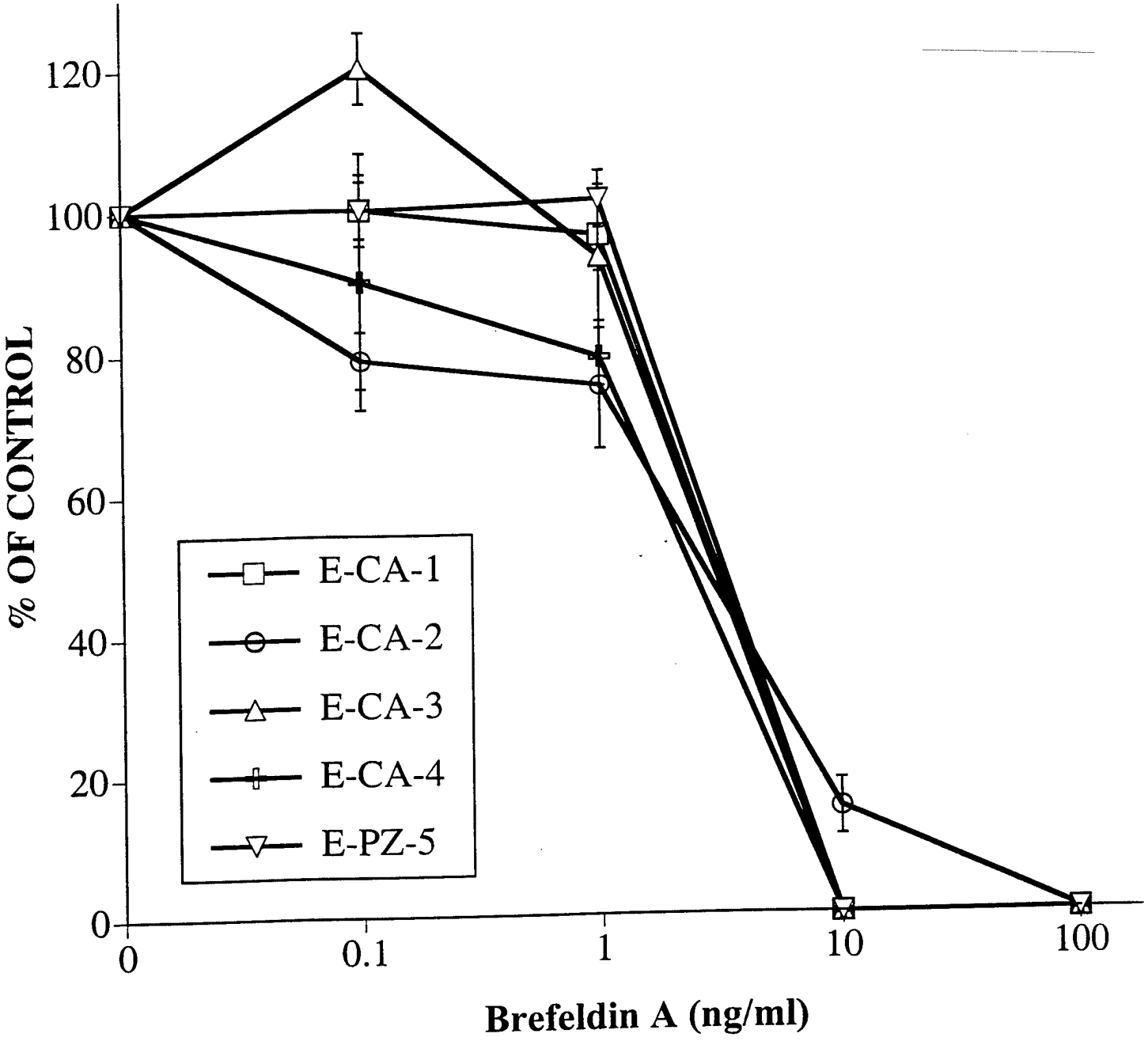
Figure 4. DNA laddering in response to BFA. E-CA-4 cells were treated with or without 25 ng/ml of BFA for 0, 8, 24 or 48 hours. DNA was extracted and electrophoresed on a 1.8%

agarose gel, followed by ethidium bromide staining. A molecular weight ladder (123 bp) was run as a marker.

Figure 5. Protein levels of p53 in response to BFA. E-CA-2 cells were treated with or without 10 ng/ml of BFA and cell lysates were prepared at 0, 3, 6, 24 and 48 hours. Immunoblots were used to detect p53 protein. Control cells were SV40-transformed prostatic epithelial cells (pRNS-1-1), which have high levels of p53 protein, and E-CA-4 cells treated with DRB for 24 hrs. PC-3 cells, which lack p53 expression, served as a negative control. Equal loading of lysates in the lanes is indicated by equivalent intensities of nonspecific bands.

Figure 6. Levels of pRB in response to BFA. E-CA-2 cells were treated with or without 10 ng/ml of BFA and cell lysates were prepared at 0, 3, 6, 24 and 48 hours. Immunoblots were used to detect pRB protein. Bands corresponding to hyper- and hypo-phosphorylated pRB are indicated; the nonspecific band at the higher molecular weight serves as a loading control and indicates equivalent loading of lysates from treated and untreated cells.

FIGURE 1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80
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% OF CONTROL
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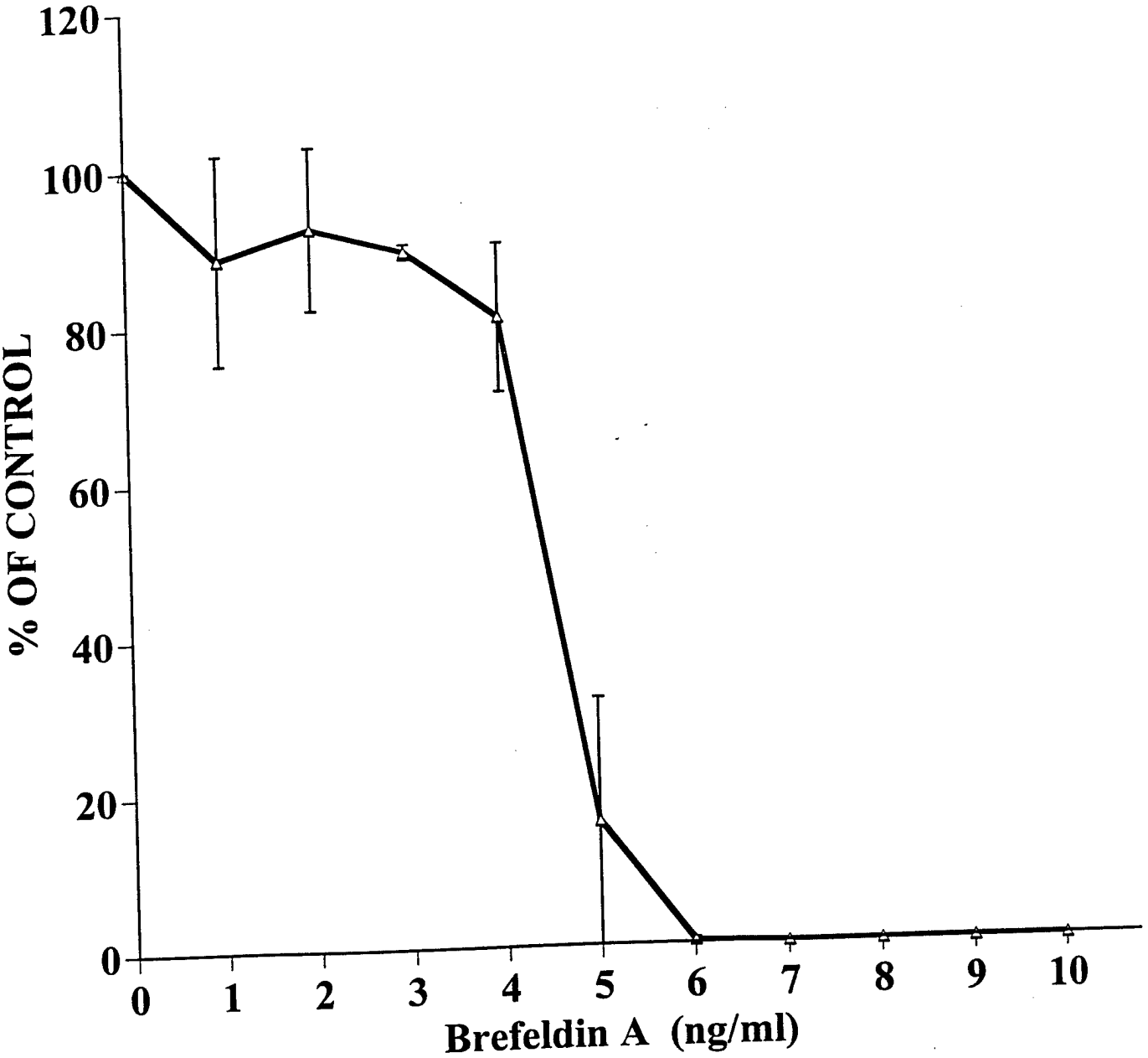
E-CA-2
E-CA-3
E-CA-4
E-PZ-5
0
0.1
1
10
100
Brefeldin A (ng/ml)

FIGURE 2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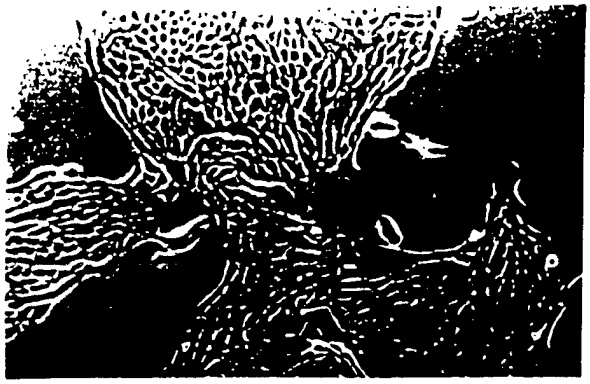
Brefeldin A (ng/ml)



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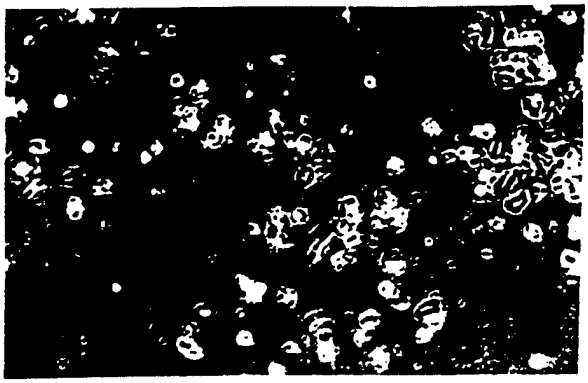
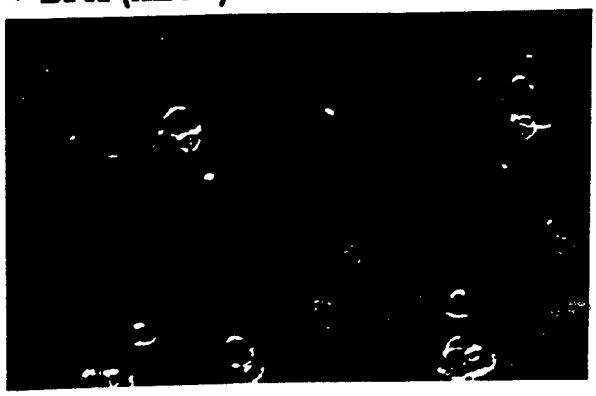


+ BFA (x200)



+ BFA (x800)

FIGURE 4

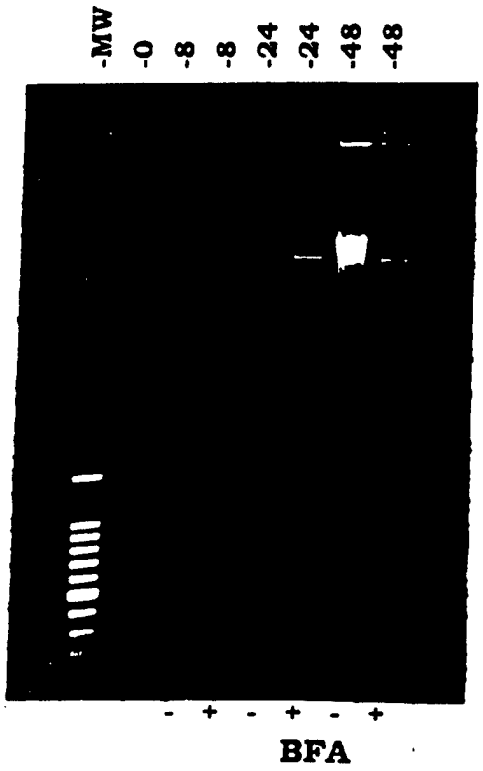


FIGURE 5

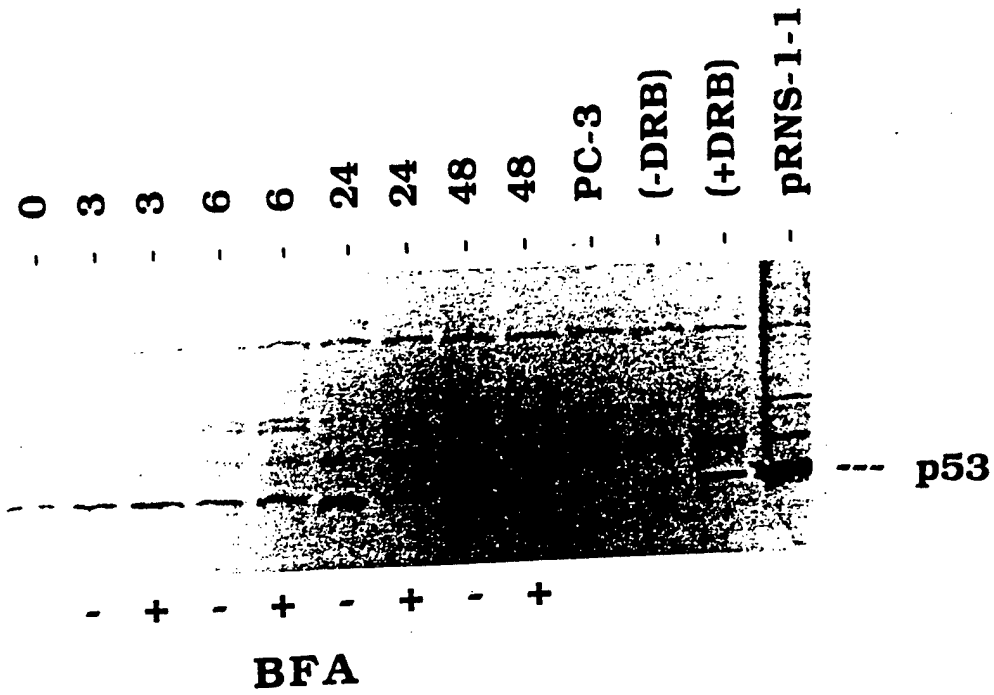
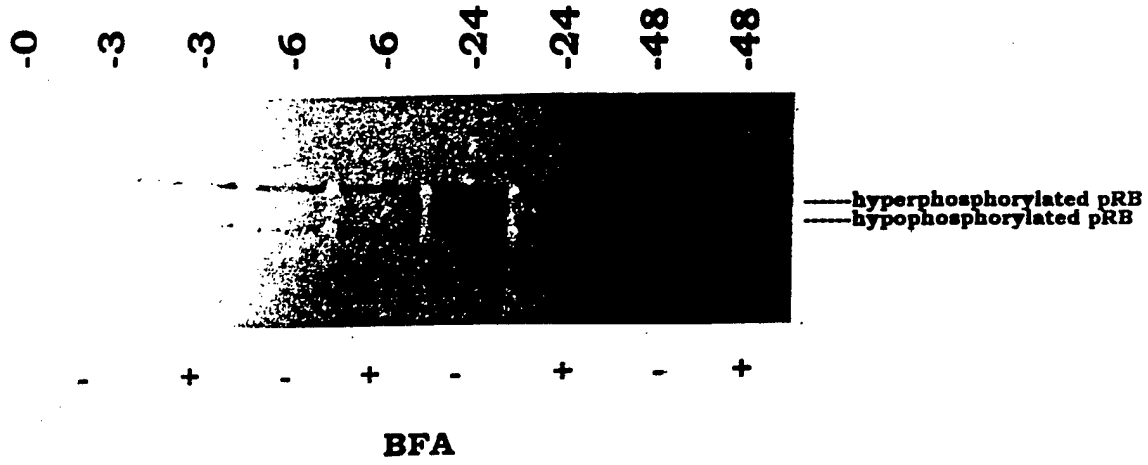


FIGURE 6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-3
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hyperphosphorylated pRB
hypophosphorylated pRB
-
+
-
+
-
+
-
+
BFA

$p16^{INK4A}$ Mediates Cdk4/6 Inhibition In Senescent Prostatic Epithelial Cells

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Running title: $p16^{INK4A}$ mediates cdk4 inhibition in senescent prostate epithelial cell senescence

Key words: Senescence, Cell Cycle, $p16^{INK4A}$, Cyclin D1/Cdk4

ABSTRACT

The senescence checkpoint constrains the proliferative potential of normal cells in culture to a finite number of cell doublings. In this study, we investigated the mechanism of cdk inhibition in senescent human prostatic epithelial cells (HPEC). Progression of HPEC from early passage to senescence was accompanied by a gradual loss of cells in S phase and an accumulation of cells with a 2N DNA content. Furthermore, G1/S phase-associated kinase activities progressively diminished with increasing cell passage. In senescent HPEC, cdk4 and cyclin E1- and A-associated kinases were catalytically inactive. In contrast to observations in senescent fibroblasts, levels of the KIP inhibitor $p21^{CIP1}$ diminished over the proliferative lifespan of HPEC. Levels of $p27^{KIP1}$ fell as cells approached senescence and the association of both $p21^{CIP1}$ and $p27^{KIP1}$ with cdk4/6 complexes was decreased. However, the level of cyclin E1-associated KIP molecules was unaltered as cells progressed into senescence. Progression to senescence was accompanied by a progressive increase in both the level of $p16^{INK4A}$ and in its association with cdk4 and cdk6. As HPEC approached senescence, cdk4- and cdk6-bound $p16^{INK4A}$ showed a shift to a slower mobility due to a change in its phosphorylation profile. As $p16^{INK4A}$ increased in cdk4 and cdk6 complexes, there was a loss of cyclin D1 binding. The altered phosphorylation of $p16^{INK4A}$ in senescent prostatic epithelial cells may facilitate its association with cdk4 and cdk6 and play a role in the inactivation of these kinases.

INTRODUCTION

The eventual growth arrest that defines the termination of cellular proliferation of normal cells in culture is referred to as cellular senescence (1). Normal cells can undergo a finite number of population doublings in culture before they stop proliferation at senescence (1, 2). Fibroblasts arrested at senescence have a predominantly 2N DNA content, reflecting arrest during the G1 phase of the cell cycle (2). Several lines of evidence suggest that the mechanisms regulating cell cycle arrest at senescence are genetically programmed and reflect processes relevant to aging within the organism (3-5). It has been postulated that the senescence checkpoint may function as a critical tumor suppression checkpoint in vivo. Estimations of cellular proliferation have suggested that tumor growth beyond a volume of $1cm^3$, requires abrogation of the senescence arrest (6).

Most previous studies investigating senescence have utilized fibroblasts. The high incidence of prostatic cancer in adult males and our limited understanding of prostatic oncogenesis motivated our study of prostatic epithelial cell senescence. Primary prostatic cancers frequently show telomerase activation (7-10) and it is possible to establish immortal lines from primary prostate cancers (11-13). Thus prostate cancer development may be associated with loss of the senescence checkpoint. An understanding of the molecular mechanisms whereby the senescence checkpoint is lost in cancers requires an assessment of this checkpoint in normal human prostatic epithelial cells (HPEC). In the present study, we investigated the mechanisms of cyclin-dependent kinases (cdk) inhibition in senescent HPEC.

Transition from one phase of the cell cycle to the next requires the orderly activation and inactivation of a family of related cyclin-dependent kinases, cdks 1 to 7 (reviewed by (14, 15)).

Cdks are activated by cyclin binding (14) and regulated by phosphorylation (16). G1 phase to S phase progression requires phosphorylation of the retinoblastoma protein (pRb) which is mediated primarily by cyclin D1-associated cdk4 or cdk6, and also by cyclin E-cdk2 (17, 18).

Two families of cdk inhibitors, the **In**hibitors of cd**k4** (INK4) and the **k**inase **i**nhibitor **p**roteins (KIP) regulate cdk activity (reviewed by (19, 20)). The KIP family consists of three broadly acting inhibitors $p21^{CIP1}$, $p27^{KIP1}$ and $p57^{KIP2}$. KIP family members bind to and inhibit the cyclin-cdk complexes. Recent in vitro experiments demonstrate that a single KIP molecule is sufficient to inhibit cyclin/cdk kinase activity (21). In contrast to the KIP inhibitors, members of the INK4 family ($p15^{INK4B}$, $p16^{INK4A}$, $p18^{INK4C}$ and $p19^{INK4D}$) bind specifically cdk4 and cdk6, with resulting loss of cyclin D binding and catalytic inactivation.

Cell culture models have identified a role for the cdk inhibitors in senescence. An increased expression of p21 and/or p16 at senescence has been identified in human and murine fibroblasts and melanocytes (22-26). The induction of p21 in senescent fibroblasts led to the initial identification and cloning of this gene by Noda et al. (27). The elevated expression of p16 and/or p21 at senescence is associated with their increased binding and inhibition of G1/S phase cdks. Elimination of *p21* expression through homologous recombination extended the lifespan of human diploid fibroblasts in culture (28). Thus, the loss of p21 expression, while itself not sufficient to abrogate senescence arrest, may represent a key step in the immortalization of cells of fibroblastic lineage. The loss of *p21* and *p16* expression in human cancers suggests that in vivo, these inhibitors may contribute to the senescence checkpoint and limit tumor development (29, 30).

In human prostatic tumors, although *p16* is rarely mutated (31, 32), loss of expression occurs frequently through hypermethylation or deletion (29, 33, 34). Furthermore, loss of p16

expression in human prostatic tumors may have prognostic implications (30). Together, these results suggest that p16 may play an important tumor suppressor role in prostatic epithelial cells. In this study, we showed that the progression toward HPEC senescence was associated with an increase in p16 levels. Novel phosphorylated forms of p16 showed increased association with target cdk complexes, cdk4 and cdk6. In contrast to senescence in fibroblasts, neither the expression of p21 nor its association with target cdk complexes was increased. Phosphorylation of p16 may represent a novel regulatory pathway for p16 inhibitory activity.

MATERIALS AND METHODS

Cell Culture. Cell cultures were established as follows: cell strains E-PZ-16 and E-PZ-22 were obtained from men aged 60 and 61 years, respectively, undergoing radical prostatectomy to treat prostate cancer. Neither had received previous therapy. Prostatic specimens were transferred to the laboratory within one hour following surgery. A small wedge of tissue was dissected from the peripheral zone of each specimen and primary cultures were established as previously described (35). Briefly, tissues were minced and digested overnight with collagenase. The digested tissues were inoculated into dishes coated with collagen type I and containing medium PFMR-4A supplemented with 10 ng/ml of cholera toxin, 10 ng/ml of epidermal growth factor, 40 μg/ml of bovine pituitary extract, 4 μg/ml of insulin, 1 μg/ml of hydrocortisone, 100 μg/ml of gentamicin, 0.1 mM phosphoethanolamine, 3 nM selenous acid, 2.3 μM alpha-tocopherol, and 0.03 nM all-trans retinoic acid (35). Cells that grew out in primary culture were aliquoted and stored frozen in liquid nitrogen. The epithelial nature of these cells was verified by immunocytochemical staining for cytokeratins (35). To verify the histology of origin, the prostatic specimens were inked after dissection, fixed, and serially sectioned (36). The histology

of tissues immediately adjacent to and surrounding the portion removed for culture was reviewed. Neither cancer or benign prostatic hyperplasia was present in the areas of tissue from which the cell strains were derived.

Cells were serially passaged as follows: cells were thawed and inoculated into collagen-coated dishes containing MCDB 105 (Sigma. St. Louis. MO) supplemented as described for PFMR-4A except with 10 rather than 40 μg/ml of pituitary extract. When approximately 50% confluent. a portion of the cells was harvested for analyses of cell cycle regulators at "passage 1". The remainder of the cells (about 10 or 20%) were passaged following trypsinization into 40 dishes and again grown to about 50% confluency. at which time cells were again harvested ("passage 2") or passaged. This process was repeated twice more (passages 3 and 4) until the cells ceased proliferation.

Flow cytometric analysis. At different passages. cells were pulse-labeled with 10 μM bromodeoxyuridine (BrdU) for 2 hours. Cells were then harvested. fixed with 70% ethanol. treated with 0.1 N HCl and heated for 10 minutes at 90^{O} C to expose the labeled DNA. Cells were then stained with anti-BrdU-conjugated FITC (Becton Dickinson. Bedford. MA) and with propidium iodide. Cell cycle analysis was carried out on a Becton Dickinson FACScan, using Cell Quest software.

Antibodies. Antibodies to the retinoblastoma protein. cdk2, cyclins A and D1, and p21 were obtained from Pharmingen (San Diego. CA) or Santa Cruz Biotechnology (Santa Cruz. CA). Cyclin E1 specific antibodies (mAbs E12 and E172) (37. 38) were from E. Lees and E. Harlow (Massachusetts General Hospital. MA). Monoclonal PSTAIRE antibody (39) was a gift from S. Reed (The Scripps Research Institute. CA). and cyclin D1 antibody. DCS-11. was purchased from Neomarkers (Fremont. CA). Cyclin A monoclonal mAb E67 was provided by J.

Gannon and T. Hunt (ICRF, England). Monoclonal p27 antibody was purchased from Transduction Labs (Lexington, KY). Cdk4 and cdk6 polyclonal sera were provided by G. Hannon and D. Beach (CSH Labs, NY). The JC-6 monoclonal provided by J.Koh and E. Harlow (40), and p16 polyclonal antibody purchased from Santa Cruz, were used for immunoblotting of p16 in these studies. β-actin antibody was purchased from Sigma. Monoclonal p19 antibody was obtained from Neomarkers. Polyclonal p18 antibody was kindly provided by Y. Xiong (Chapel Hill, NC).

Immunoblotting. Cells were lysed in ice cold NP-40 lysis buffer (0.1% NP-40, 50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM phenyl methyl sulfonyl fluoride and 0.02 mg/ml each of aprotinin, leupepsin and pepstatin). Lysates were sonicated and clarified by centrifugation.

Protein was quantitated by Bradford analysis. Twenty or 50 μg of protein were loaded in each lane and resolved by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (SDS-PAGE). Transfer and blotting was as described (41). Equal loading of the lanes was verified using a β-actin antibody. For detection of cdk4-associated proteins by immunoprecipitation/Western analysis (IP/Western), cdk4 was immunoprecipitated from 200 μg of protein lysate, complexes resolved, blotted and the blot reacted with either cdk4, cyclin D1, p16, p21 or p27 antibodies. To verify the identity of associated proteins, control cyclin D1, p16, p21, and p27 immunoprecipitations were resolved along side the cdk4 immune complexes (not shown). Similar methods were used to detect cdk6 and cyclin E1- associated proteins.

Protein expression in bacteria. pRb substrate for cyclin D1-associated-kinase assays was generated from a pGEX vector containing the C-terminus of pRb (amino acids 729 to 928) fused to GST (kindly provided by J. Zhao and E. Harlow, Massachusetts General Hospital, MA). Bacteria were lysed in phosphate-buffered saline PBS containing 1% Triton X-100, 1 mg/ml lysozyme and protease inhibitors, and lysates were clarified by centrifugation at 15K RPM for 20 minutes. The pRb fragment was isolated by incubating bacterial extract with glutathione beads

for 1 hour. Beads were then washed repeatedly with PBS 1% Triton X-100 and then with PBS alone. The pRb fragment was eluted in 20 mM glutathione PBS at pH 7.8.

Kinase Assays. For cdk4 kinase assays, cells were lysed in 50 mM Hepes . 150 mM NaCl. 1 mM EDTA pH 8.0. 2.5 mM EGTA pH 8.0. 10% glycerol. 10 mM β-glycerophosphate. 1mM NaF. 0.1% Tween-20. 0.1 mM Na_2VO_4. 1mM AEBSF [4-(2-aminoethyl) benzenesulfonylfluoride], 0.5 mM dithiothreitol (DTT) and 1 mg/ml of both leupeptin and aprotinin. Cdk4 kinase assays were performed following the method of LaBaer et al. (42) using cdk4 antibody obtained from Santa Cruz for immunoprecipitation and the carboxyl-terminal fragment of pRb as substrate. Quantitation of radioactivity was performed using a Molecular Dynamics PhosphorImager and ImageQuant software. Cyclin E1- and A-associated kinase assays were performed as described (43). Histone H1 substrate was obtained from Boehringer Mannheim (Laval, Quebec). Background levels of kinase activity were determined for each kinase reaction by immunoprecipitating early passage cellular extract with non-specific mouse monoclonal antibodies (labeled as lane c in Fig. 3).

Phosphatase Assay. Dephosphorylation of p16 was carried out by immunoprecipitating cdk6 complexes from 100 µg of cellular extract. Complexes were washed three times with NP-40 lysis buffer and twice with potato acid phosphatase (PAP) buffer [40 mM PIPES pH 6.0 (Piperazine-N,N'-bis[2-ethanesulfonic acid]) (Sigma), 1 mM DTT, 20 µg/ml aprotinin and 20 µg/ml leupeptin]. An ammonium sulfate precipitate of PAP (Sigma) was resuspended in 1 ml of PAP buffer and eluted from a desalting column (Pharmacia Nap-5) with 500 µl of PAP buffer. Immunoprecipitated complexes were then incubated with 12 U of potato acid phosphatase for 2 hours. The specificity of the phosphatase assay was confirmed by incubating

immunoprecipitates with 1 mM Na_2VO_4 and 100 mM β-glycerophosphate in addition to PAP. The complexes were then resolved by SDS-PAGE and immunoblotted for p16.

Two-Dimensional Gel Electrophoresis. Isoeletric focusing of p16 was carried out by immunoprecipitating cdk6 complexes from 1 mg protein cell extract. The immune complexes were washed three times with NP-40 lysis buffer and then once with 20 mM Tris pH 7.5. The washed complexes were solubilized in 200 µL of Rehydration Buffer (8 M urea, 2% (w/v) CHAPS (3-[3-(choloamidopropyl)]-dimethyl-[ammonio]-1-propanesulphonate), 0.5% (v/v) pH 3-10 IPG (Immobiline Dry Strip Gels) buffer (Amersham Pharmacia Biotech) and 18 mM DTT) and loaded onto IPG strip holders containing pH 3-10 linear IPG strips (Amersham Pharmacia Biotech). The gels were rehydrated with the sample solution for 17 hours at 20°C, and then focused consecutively for 1 hour at 500 V, 1 hour at 1000 V and for 2 hours at 8000 V in an IPGPHOR Isoelectric Focusing System (Amersham Pharmacia Biotech). The focused gel strips were incubated for 15 minutes at room temperature in Equilibration Buffer (50 mM Tris pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS and 65 mM DTT) prior to loading onto 17.5 % SDS-PAGE gels. The electrophoresed gels were then transferred to immobilon and p16 detected by immunoblotting.

RESULTS

Cell cycle arrest in senescence. The cell cycle profile of HPEC was determined by BrdU pulse labeling and FACs analysis of cells at early passage and senescence (Fig. 1). At early passage, 22% of asynchronously growing cells were in S phase, 66% contained a 2N DNA content and the remaining 12% of cells contained a 4N DNA content. Cellular proliferation ceased after 6 passages, which corresponded to approximately 30 population doublings at

doublings was largely due to loss of the CAK-activated, thr-160 phosphoform (faster migrating cdk2 band).

The levels of the KIP and INK inhibitors were also analyzed in asynchronous prostatic epithelial cells at increasing population doublings and at senescence. There was a steady decrease in the protein levels of $p21^{CIP1}$ and $p27^{KIP1}$. $p18^{INK4C}$ expression was not detected (not shown) and $p19^{INK4D}$ levels remained unchanged, while $p16^{INK4A}$ levels steadily increased, reaching maximal levels of expression in senescent cells. In addition, longer exposures of the p16 immunoblots revealed the presence of two additional p16-reactive bands of decreased mobility on SDS-PAGE (Fig. 3B). These slower mobility bands were detected using two different anti-p16 antibodies (the JC-6 monoclonal and the Santa Cruz polyclonal). The abundance of these two novel p16-related bands increased with increasing population doublings.

KIP binding to cyclin E1/cdk2 did not increase in senescent HPEC. The composition of cyclin E1/cdk2 complexes showed no apparent change during the progression of HPEC toward senescence (Fig. 4A). The amount of cdk2 bound to cyclin E1 was not reduced. Furthermore, the proportion of thr-160 phosphorylated cdk2 bound to cyclin E1 remained constant. The levels of cyclin E1-associated p21 and p27 remained constant. Thus, the inhibition of cyclin E1/cdk2 activity could not be attributed to increased KIP binding or to a lack of activating phosphorylation at the thr-160 residue of cdk2.

p16 accumulated in cdk4 and cdk6 complexes as HPEC approached senescence. As total $p16^{INK4A}$ levels increased during the aging of the HPEC population, the association of $p16^{INK4A}$ with cdk4 and cdk6 complexes also increased (Fig. 4B, C). Although there was no increase in the faster mobility band of p16 in cdk4 and cdk6 complexes (Fig. 4B, C short exposure), longer exposures of the cdk-associated p16 immunoblots (Fig. 4B, C long exposure)

senescence. Cells were considered to be senescent after they remained sub-confluent for more than 1 month. Flow cytometry of senescent HPEC demonstrated a cell cycle arrest with less than 1% of cells in S phase and 85% of the cells with a 2N DNA content. The remaining 14% of cells had a 4N DNA content at senescence.

Cdk activities decreased progressively with increasing passage. The kinase activities in cyclin E1-, cyclin A- and cdk4 immune complexes from cell populations of increasing passage were assayed using either histone H1 (cyclin A, E) or a pRb protein fragment (cdk4) as substrates. Results are shown in Fig. 2. The activities of cyclin E1- and cyclin A-associated kinases and of cdk4 decreased steadily as cells progressed from early passage to senescence. Senescent cells showed no kinase activities above that in non-specific immune controls. Furthermore, analysis of cells of increasing passage revealed a progressive loss of pRb phosphorylation, indicative of cell cycle arrest in G1 (see Fig. 3A). Since pRb is phosphorylated by cyclin E1- and D1 associated kinases, loss of pRb phosphorylation provides further evidence of inhibition of these cdks.

Increased $p16^{INK4A}$ levels in senescent prostatic cells. The levels of cdk inhibitors, cyclins and cdks associated with the G1/S transition were assayed as HPEC progressed from early passage toward senescence (Fig. 3A). Western analysis revealed no change in cyclin E1 or cyclin D1 levels during the aging of prostatic epithelial cell populations. Cyclin A levels gradually decreased, consistent with the gradual recruitment of cells into G1 arrest at senescence. The decrease in cyclin A levels was likely the consequence of decreased cyclin E1- and D-associated kinase activities, resulting in reduced E2F-mediated transcription of cyclin A. The cdk4 and 6 levels remained unchanged. The loss of cdk2 protein with increasing population

again revealed the existence of two delayed mobility bands that cross-reacted with p16 specific antibodies. The association of these two bands with cdk4 and cdk6 complexes steadily increased with later cell passages. Densitometry showed that association of the slower migrating forms of p16 increased by 2 fold in cdk6 complexes and by 1.6 fold in cdk4 complexes between passage 2 and senescence. The accumulation of these slower mobility p16 bands in cdk4 and cdk6 complexes was correlated with a decrease in the binding of cyclin D1, p27, p21 and p19. None of the anti-p16 reactive bands were cross-reactive with p18 or p19 antibodies.

Altered phosphorylation of p16 in senescent prostatic epithelial cells. To determine whether the novel bands of delayed mobility were different phosphoforms of p16, cdk6 complexes were immunoprecipitated from senescent cellular extract (Fig. 5, lane 1) and treated with potato acid phosphatase (PAP) (lane 2), and immunoblots reacted with p16 antibodies. PAP treatment of the immunoprecipitates from senescent cells resulted in the loss of the upper two p16 bands, visible on long exposures, and the formation of two bands of increased mobility (data for senescent cells shown in lower 2 panels, Fig. 5). Thus, the slower mobility forms of p16, whose association with both cdk4 and cdk6 complexes increased in senescent HPEC, represent novel phosphoforms of p16. These delayed mobility p16 phosphoforms were not detected, even with prolonged exposure in the early passage HMEC. It is notable that the dominant, faster mobility p16 band, detected in both early passage and senescent cells, also shifted to two faster mobility bands when the phosphatase reaction went to completion, indicating that this dominant band also represents phosphorylated p16 (see upper two panels, Fig. 5). There was no loss of any of the p16 reactive bands when PAP was pre-incubated with phosphatase inhibitors (lane 3). The formation of two p16 reactive bands of increased mobility upon PAP treatment suggested

that all of the cellular p16 is phoshorylated, but the pattern of expression of the different phosphoforms differed between early passage and senescent cells.

Two-dimensional isoelectric focusing (2D IEF) of p16 in cdk6 immunoprecipitates with and without PAP confirmed the existence of multiple p16 phosphoforms (Fig. 6). In early passage cells, the solitary band of p16 seen on one-dimensional western blots (Fig. 4) resolved on 2D IEF as two forms of p16, which focused to a pH between 5 and 6 (see arrow 3, Fig. 6). In senescent cells, in addition to the two forms of p16 observed in early passage cells, two additional p16 isoforms (labeled 1 and 2 in Fig. 6) were present. These two isoforms showed a delayed mobility on SDS PAGE (the second dimension) and focused just below pH 6.0. The relative mobilities of isoforms 1 and 2 in the second dimension with respect to the predominant forms of p16 was consistent with these being the two minor p16 bands of delayed mobility that were observed previously in one-dimensional western blots in Fig. 4.

With PAP treatment prior to their resolution on 2D IEF, all of the p16 isoforms focusing between pH 5.0 and 6.0 were lost. Phosphatase treated p16 focused at a higher isoelectric point, at approximately pH 6.8 and was of increased mobility in the second dimension. These results confirm the results in Fig. 5 and indicate that all three p16 bands detected on western blotting represent p16 phosphoforms. The pattern differs between early passage and senescence, with isoforms 1 and 2 increasing as cells approach and enter senescence.

DISCUSSION

The senescence checkpoint limits the proliferative capacity of cells in a tissue culture environment. Processes analogous to the senescence checkpoint observed in cultured cells are thought to limit cellular proliferation in vivo. A number of observations suggest that senescence

occurs within the organism in vivo. The proliferative capacity of cells with finite lifespan in culture is inversely related to the donor's age (3). The number of population doublings that fibroblasts undergo in culture is related to the longevity of the donor species (4). The limit imposed by senescence on the proliferative capacity of cells has raised the hypothesis that cellular senescence may indeed represent a natural impediment to malignant degeneration. To date, most studies investigating senescence have utilized human fibroblasts and rodent cells. Since the incidence of malignancies arising from fibroblasts such as sarcomas is relatively rare in humans, we have examined the senescence phenomenon in epithelial cells, which undergo malignant transformation more frequently than fibroblasts. Our investigation of senescence arrest in human prostatic epithelial cells and in mammary epithelial cells (unpublished results) demonstrates that epithelial cells differ from fibroblasts in the manner by which cdk inhibition occurs at senescence.

The identification of key inhibitor(s) of cell cycle progression at senescence could potentially indicate a critical regulator whose expression or activity would need to be down regulated for tumor progression to proceed. Investigations of senescence in fibroblasts suggest important roles for p21 and p16. Here we have demonstrated an increase in p16 and the appearance of novel phosphorylated forms of p16 that bind and inhibit cdk4 and cdk6 activity in senescent HPEC.

The prostatic epithelial cell cultures used in this study moved from a proliferatively active state to a senescent state within six passages or approximately 30 population doublings. Senescent HPEC showed a predominantly 2N DNA content. A small proportion of cells had a 4N DNA content at senescence. We and others have observed an increase in tetraploidy as cultured cells approach senescence (44, Sandhu et al. [unpublished results]). Thus, cells with a

4N DNA content are most likely tetraploid cells arrested prior to S phase entrance at the senescence checkpoint, as opposed to cells arrested at the G2/M transition. The catalytic activity of the cdks associated with the G1/S transition underwent a steady decrease from early passage to senescence. The steady loss of kinase activity implies that the entry of cells into senescence does not occur in a synchronized manner. Rather, there appears to be some heterogeneity with regard to the passage at which epithelial cells enter senescence. Early studies of senescence identified this phenomenon by the progressive reduction of tritiated-thymidine incorporation that occurred during the serial passaging of fibroblasts (45). The established relationship between telomere length and the proliferative capacity of cells suggests that variations in telomere length within a population of cells may account for the apparently stochastic manner whereby cells enter senescence (46). The heterogeneity in telomere length may reflect variations in the proliferation of epithelial stem cell populations in vivo.

To investigate the cause of cdk inhibition in senescent HPEC, the steady state levels of G1/S-associated cell cycle regulators were examined. Of the cyclins examined, only cyclin A diminished with increasing passage, accounting for the corresponding loss of cyclin A-associated kinase activity. The loss of cyclin A in senescent HPEC is likely a consequence of the cells arresting at a point within the cycle arrest prior to cyclin A induction. Studies in human fibroblasts have shown that entrance into senescence is similarly associated with a loss of cyclin A (47). However, in senescent fibroblasts there is a significant increase in the expression of cyclins D1 and E1 (48, 49). The levels of cdk4 and cdk6 were unaffected by increasing passage in HPEC, while loss of cdk2 was largely due to loss of the thr-160 phosphorylated form of cdk2. Fibroblasts have a similar loss of the thr-160 form of cdk2 during senescence (48, 50), and in addition, studies of fibroblast senescence have revealed a reduction in cdk4 levels (49). Thus, it

appears that the regulation of both cyclin and cdk expression differs between senescent fibroblasts and prostatic epithelial cells.

In contrast to reports in cells of fibroblastic and melanocytic lineage, the steady state levels of both p21 and p27 proteins decreased as HPEC moved toward senescence (22, 27, 51). Furthermore, there was no increase in the binding of p21 and p27 to cyclin E1-associated complexes in senescent HPEC extracts. These KIP molecules were also lost from cdk4 and cdk6 complexes during the progression towards senescence. These results contrast with previous findings in senescent human fibroblasts and keratinocytes, where p21 was shown to be induced at senescence and its binding to G1/S-associated cdks was increased (28, 51, 52). Our findings in senescent HPEC and human mammary epithelial cells (unpublished results), indicate that p21 does not appear to mediate cdk inhibition in all senescent epithelial cells. The lack of p21 induction in senescent prostatic and mammary epithelial cells may represent a fundamental difference between these epithelial cell types and keratinocytes and fibroblasts at senescence.

Investigations of senescence in fibroblasts, lymphocytes and uroepithelial cells have implicated p16 in the inhibition of cyclin D1-dependent kinases (22, 26, 53, 54). The high incidence of *p16* inactivation in prostatic tumors suggests that p16 may play a role in arresting HPEC at senescence both in vivo and in vitro (29, 33). The present study and the recent report of Jarrard et al. (55) establish that there is an increase in p16 levels in senescent prostatic epithelial cells. Immortal HPEC derivatives, generated by the introduction of HPV E6 and/or E7, were associated with a loss of p16 or pRb expression (55). We have identified two novel p16-related bands of delayed mobility that accumulate in senescent HPEC. Resolution on both one-dimensional gels and by 2D IEF after treatment with potato acid phosphatase demonstrates that the two novel p16 bands of delayed mobility observed in one-dimensional western blots

were p16 phosphoforms. In both early and late passage cell extracts. PAP treatment also shifted the most abundant (faster mobility) p16 band on the one-dimensional westerns. suggesting that all detectable p16 is phosphorylated. The failure of p16 to resolve into a single band upon prolonged PAP treatment may be a consequence of phosphorylation at sites on p16 which are not recognized efficiently by PAP or alternatively. a fraction of the cellular p16 may undergo other postranslational modifications such as glycosylation. which serve to alter its gel mobility.

PAP treatment and two-dimensional isoelectric focusing confirmed the existence of different p16 phosphorylation patterns in early passage and senescent HPEC. The dominant p16 band seen on immunoblots from both early passage and senescent cdk6 immune complexes was comprised of two p16 isoforms. Two additional p16 isoforms of delayed mobility consistently appeared in senescent cells. suggesting that p16 undergoes senescence-specific post-translational modifications. These senescent-specific isoforms (arrows 1 and 2. Fig. 6) of p16 focused at a slightly higher pH. and thus represent p16 isoforms with a lower of level of phosphorylation when compared to the two dominant isoforms of p16 (arrow 3). Following PAP treatment. all of the p16 isoforms focused at a higher pH. These results are consistent with the interpretation that all of the detected p16 is phosphorylated but that novel phosphoforms appear in senescent cells.

The increased expression of p16 translated into an increased association of p16 with cdk4 and cdk6 complexes in senescent HPEC. In senescent HPEC, as in other forms of G1 arrest, the accumulation of this INK4 molecule in target kinases was associated with loss of cyclin D1, and KIP binding (56. 57). However. only the senescence specific phosphoforms of p16 showed an increased binding to cdk complexes. The accumulation of these novel p16 phosphoforms in cdk4 and cdk6 complexes suggests a senescence-activated mechanism of post-translational modification of p16 contributing to kinase inhibition and senescence arrest in HPEC.

Phosphorylation of p16 may represent an important mechanism of p16 regulation. p16 phosphorylation may regulate either the affinity for cdk4 and cdk6 and/or the localization of p16 within the cell. Phosphorylation of p27 functions to regulate the stability of the protein and its affinity for cdk complexes (58-60). It is tempting to postulate that phosphorylation of specific sites on p16 in senescent HPEC facilitates the binding of p16 to target cdk complexes and contributes thereby to G1 arrest in senescence. The identification of these phosphorylation sites and the pathways that influence phosphorylation of p16 would aid in the understanding of the regulation of the INK family of inhibitors and elucidate further the pathways regulating p16 inhibitory activity.

ACKNOWLEDGEMENTS

J. Slingerland is a physician scientist supported by Cancer Care Ontario and by career awards from the U.S. Army Breast Cancer Program, and by the Burrough's Welcome Fund. This work was supported by grants to JMS from the U.S. Army Prostate Cancer Research Program and the Canadian Breast Cancer Research Initiative of the NCIC and to D.M. Peehl from the U.S. Army Prostate Cancer Program.

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FIGURE LEGEND

FIG. 1. Cell cycle arrest in senescence. Asynchronously growing and senescent cells were pulse-labeled with BrdU and counterstained with propidium iodide. BrdU incorporation and PI uptake were plotted for both early passage and senescent cells.

FIG. 2. Cdk/cyclin-dependent kinase activities. Cdk- or cyclin-associated kinase activities were assayed in immunoprecipitates recovered from cell lysates from early passage (1) to senescence (4). Background levels of kinase activity were determined by immunoprecipitating with non-specific mouse monoclonal antibodies from early passage cellular extracts (lane c). Reaction products were resolved by SDS-PAGE, dried and autoradiographed. Phosphorylation of substrate was quantitated by phosphoimager and the results graphed as a percentage maximum of early passage kinase activity. (A) Cdk4-associated kinase activity. The C-terminal domain of pRb was used as substrate in cdk4-associated kinase assays. (B) Cyclin E1-associated kinase activity. (C) Cyclin A-associated kinase activity. For (B) and (C), histone H1 kinase activity was assayed in cyclin E or cyclin A immunoprecipitates recovered from increasing passages.

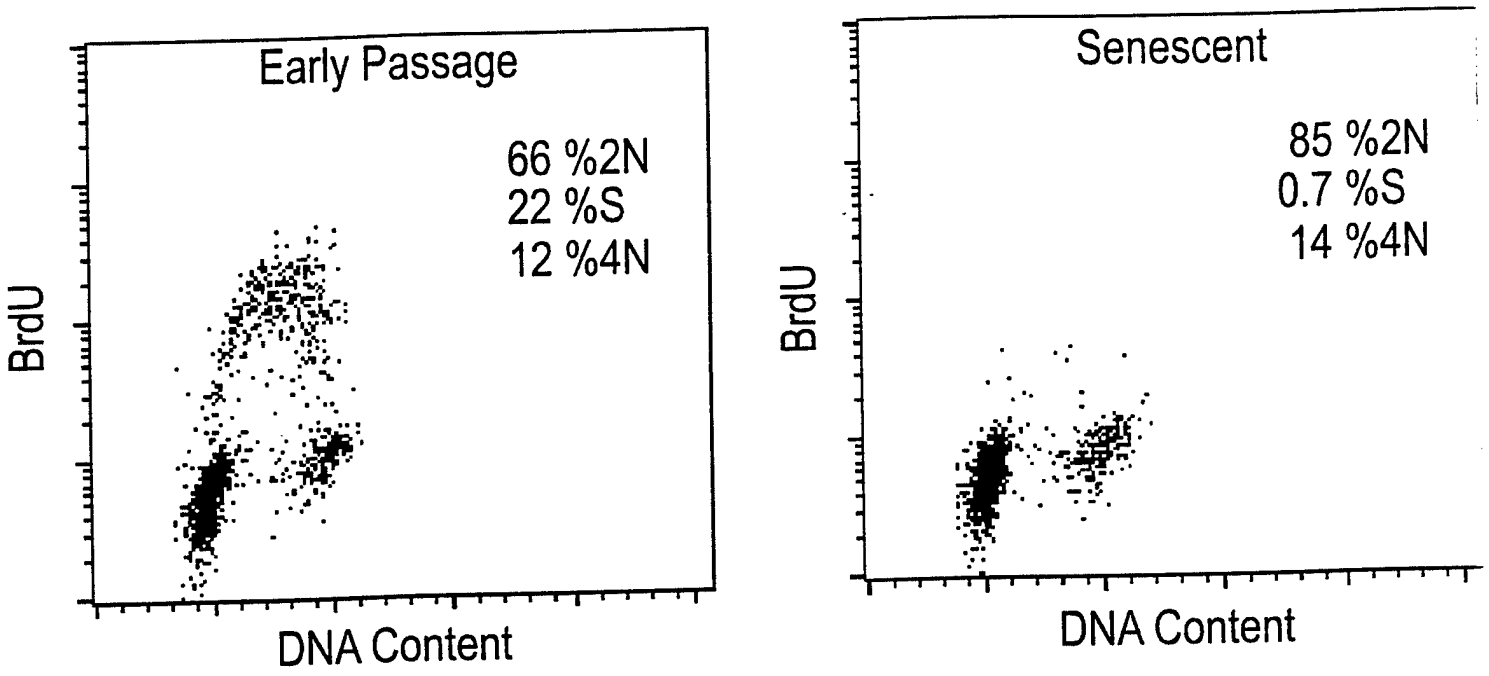
FIG. 3. Steady state levels of G1/S-associated cell cycle regulators during the aging of the prostatic epithelial cell population. (A) Lysates were collected from prostatic epithelial cells between early passage (1) and senescence (4). Cell lysates from the indicated passage numbers were resolved by SDS-PAGE and immunoblotted with the indicated antibodies. (B) Longer exposure of p16 immunoblot from (3A).

FIG. 4. Cyclin/cdk inhibitor complexes in prostatic epithelial cells from early passage to senescence. Cdk or cyclin immunoprecipitations were carried out as described in Material and Methods (A to C). Complexes from the indicated passages were resolved and immunoblotted for associated cdk, cyclin and cdk inhibitors. (A) Cyclin E1 binding to cdk2 and cdk inhibitors p21 and p27. (B) Cdk4 binding to cyclin D1 and cdk inhibitors p16, p19, p21 and p27. (C) Cdk6 binding to cyclin D1 and cdk inhibitors p16, p19, p21 and p27.

FIG. 5. p16 phosphorylation in senescent cells. Cdk6 complexes from early passage and senescent cellular extracts were immunoprecipitated and treated with potato acid phosphatase (PAP) in the presence or absence of β-glycerophosphate. Complexes were then resolved and immunoblotted for p16.

FIG. 6. Two-dimensional isoelectic focusing of cdk6-associated p16. Cdk6 complexes from early passage and senescent extracts were immunoprecipitated and treated (or not) with PAP as indicated. Complexes were resolved in the first dimension by isoelectric focusing and in the second dimension by SDS PAGE gel electrophoresis. Gels were transferred and p16 immunoblotted.

Figure 1


Early Passage
66 %2N
22 %S
12 %4N
BrdU
DNA Content
Senescent
85 %2N
0.7 %S
14 %4N
BrdU
DNA Content

A

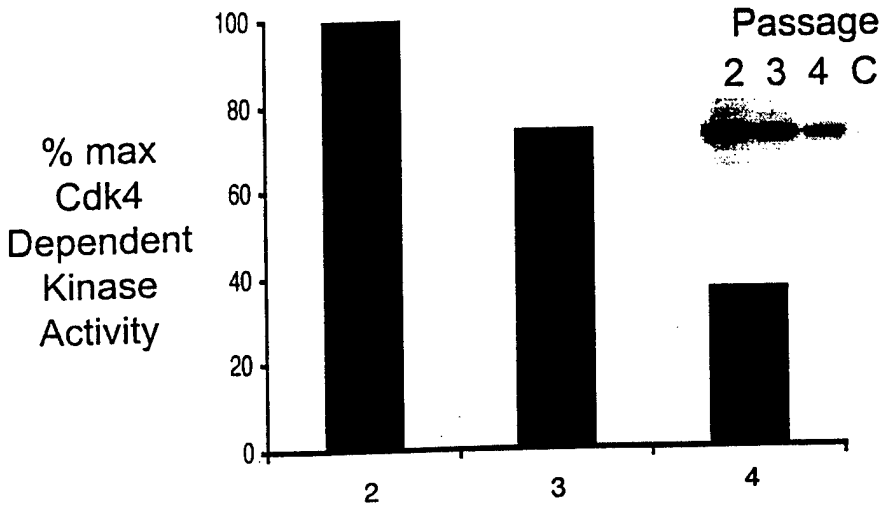
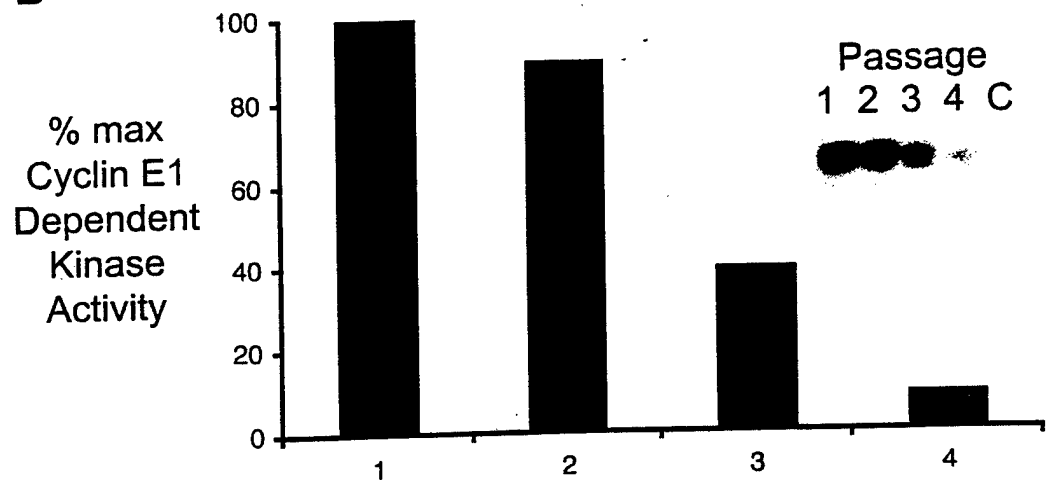

% max Cdk4 Dependent Kinase Activity
100
80
60
40
20
0
2
3
4
Passage
2 3 4 C

Figure 2

B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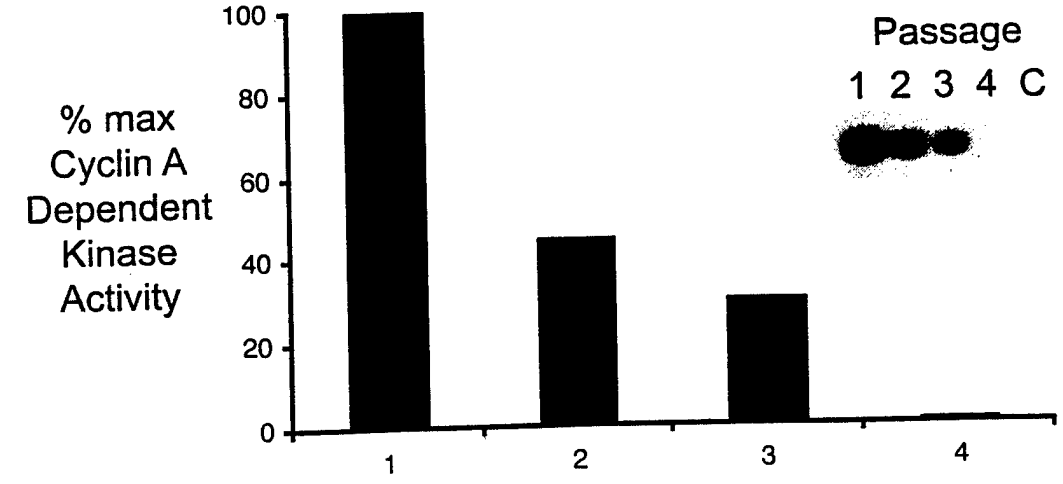
100
80
60
40
20
0
1
2
3
4
Passage
1 2 3 4 C

C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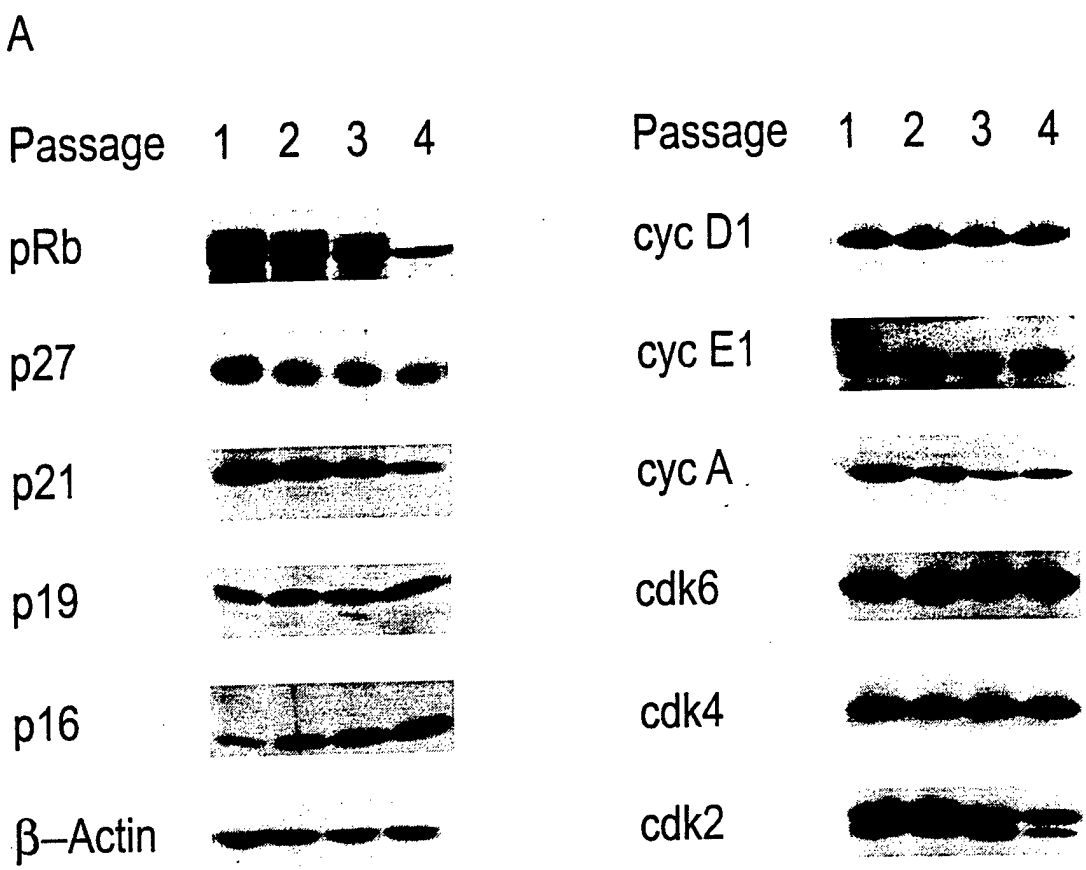
100
80
60
40
20
0
1
2
3
4
Passage
1 2 3 4 C

Figure 3


A
Passage 1 2 3 4
pRb
p27
p21
p19
p16
β–Actin
Passage 1 2 3 4
cyc D1
cyc E1
cyc A
cdk6
cdk4
cdk2
B
Passage 1 2 3 4
p16

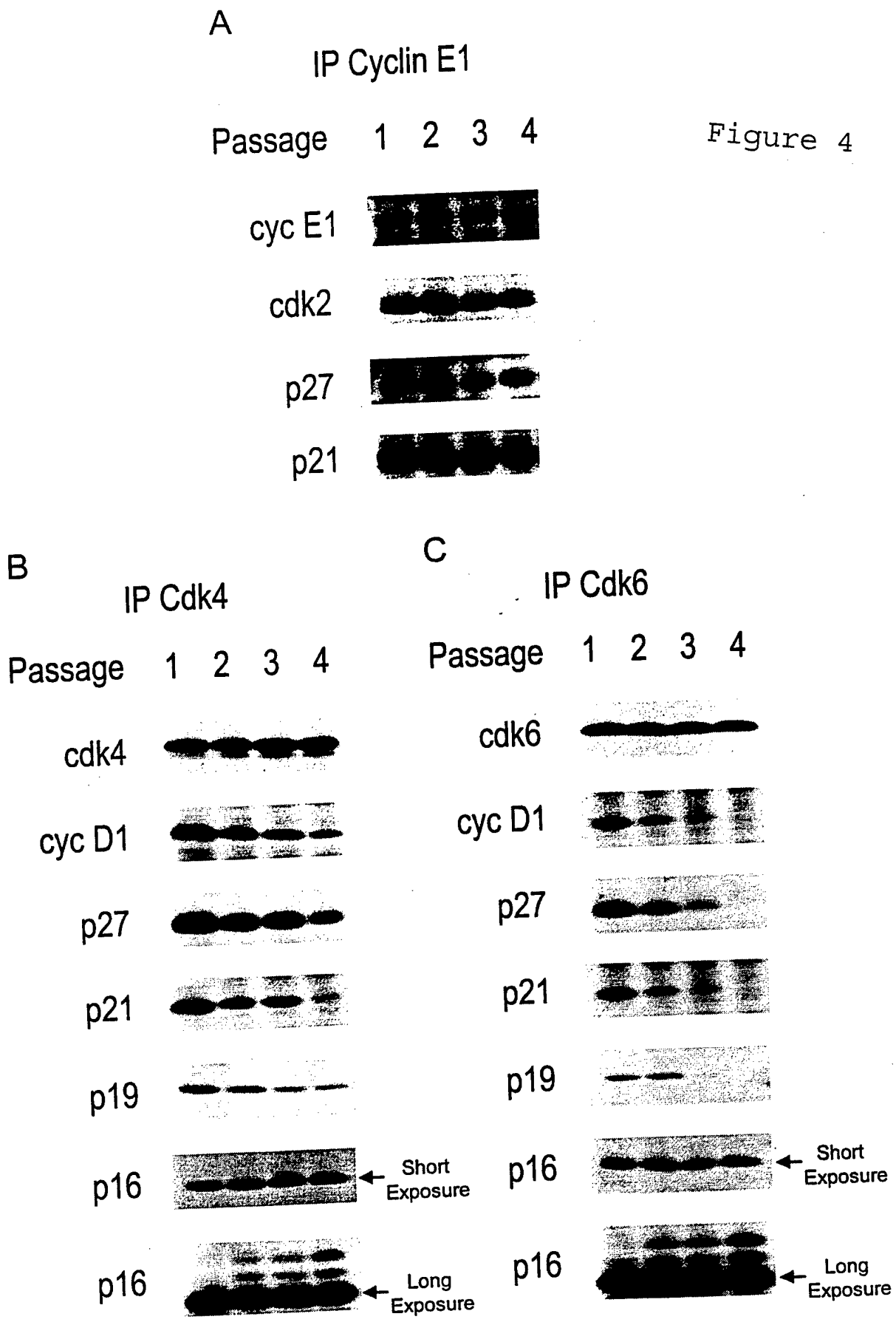

A
IP Cyclin E1
Passage 1 2 3 4
cyc E1
cdk2
p27
p21
B
IP Cdk4
Passage 1 2 3 4
cdk4
cyc D1
p27
p21
p19
p16
Short Exposure
p16
Long Exposure
C
IP Cdk6
Passage 1 2 3 4
cdk6
cyc D1
p27
p21
p19
p16
Short Exposure
p16
Long Exposure

Figure 4

Figure 5

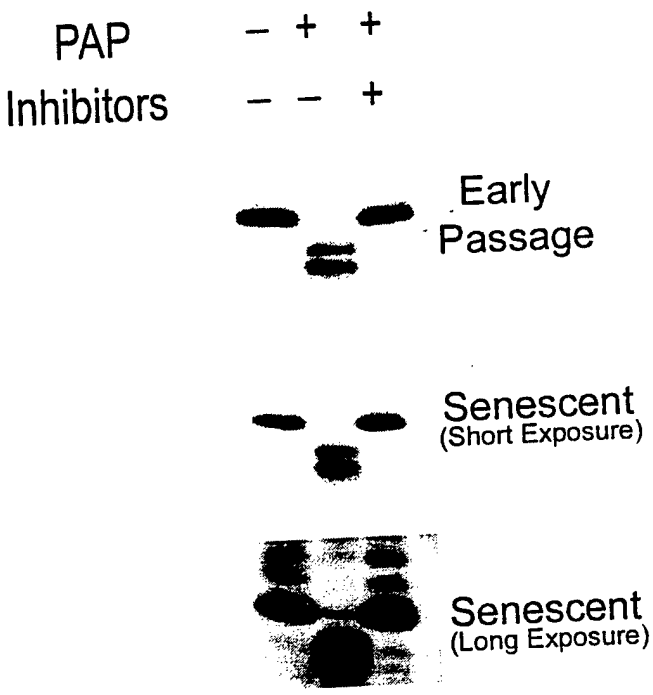
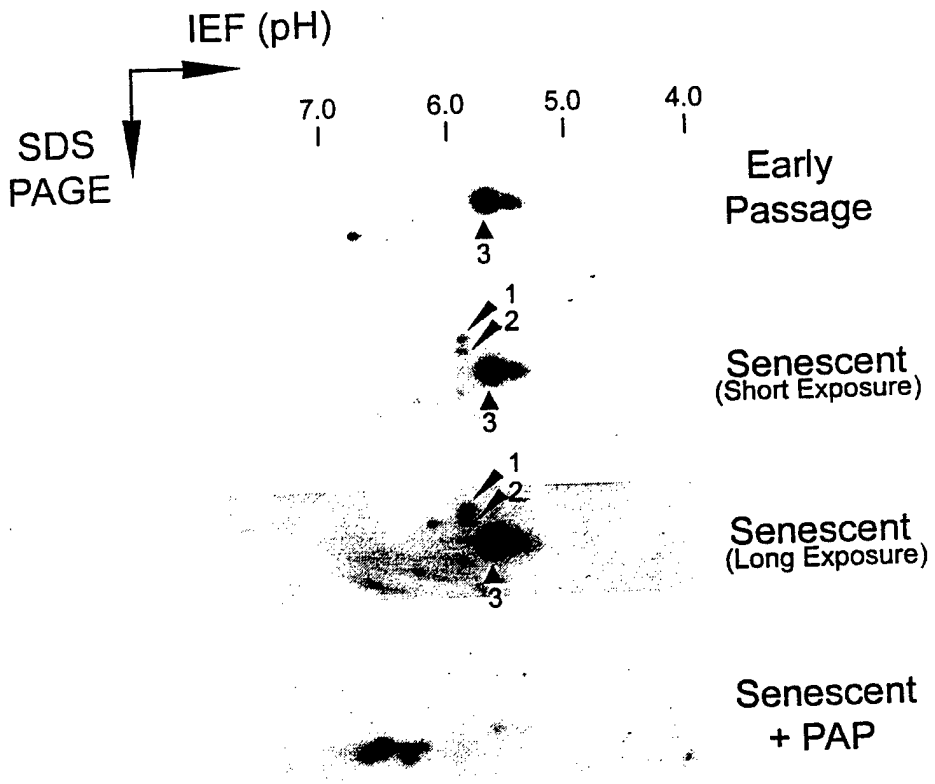

PAP
Inhibitors
– + +
– – +
Early
Passage
Senescent
(Short Exposure)
Senescent
(Long Exposure)

Figure 6


IEF (pH)
7.0
6.0
5.0
4.0
SDS
PAGE
Early
Passage
3
1
2
Senescent
(Short Exposure)
3
1
2
Senescent
(Long Exposure)
3
Senescent
+ PAP